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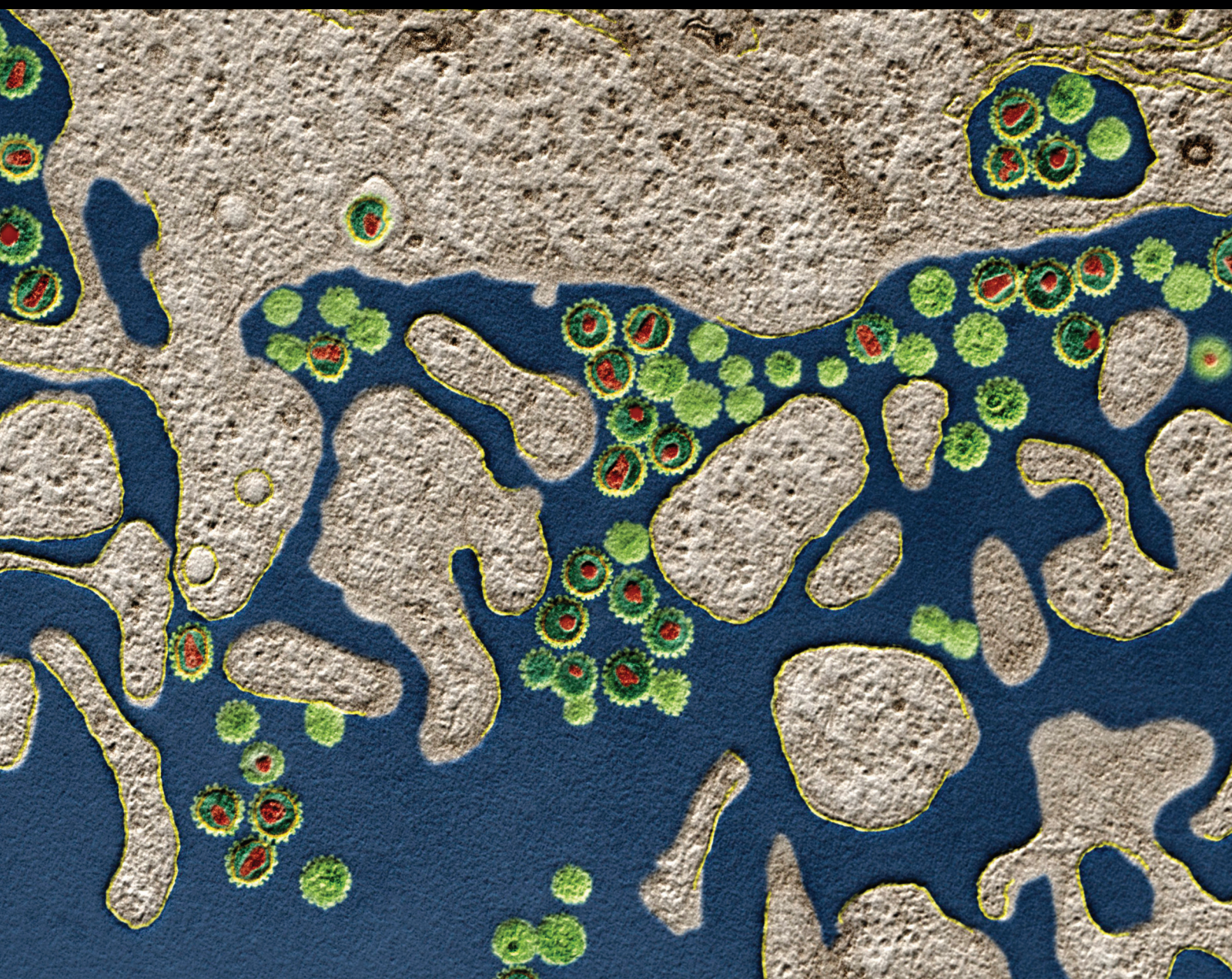
Mechanisms of Extracellular Immunomodulation Mediated by Infectious Agents

Guest Editors: Abel Viejo-Borbolla, Hans-Gerhard Burgert, and Frank A. Schildberg



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Editorial Board

Contents

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Editorial

Mechanisms of Extracellular Immunomodulation Mediated by Infectious Agents

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Secreted proteins and proteins exposed on the cell surface, referred to here together as extracellular proteins (EPs), are essential in many biological processes, including development, homeostasis, inflammation, cancer, and pathogen-host interaction. EPs comprising both host and pathogen proteins are determinants of the outcome of infection. Therefore, it is of paramount importance to discover and further characterize these proteins and their interactions to better understand the infection process and pathogenesis and to develop novel therapeutic strategies.

In this "special issue," we assembled a group of reviews and original research articles that deal with novel technologies for discovery of EPs involved in pathogen-host interaction, discuss the role of classical innate immune cytokines during viral infection, and devise novel therapeutic strategies using viral receptors as a target.

Despite the abundance and relevance of EPs, the identification and characterization of interactions with proteins of infectious agents is rather low when compared with that of intracellular proteins. Therefore, identifying and characterizing protein–protein interactions (PPI) between EPs from the host and infectious agents remain challenging and important tasks.

The recent development of novel technologies is facilitating this endeavor considerably. In her review, N. Martinez-Martin discusses classical and novel approaches to discover PPIs and explains their application to the identification of PPI between infectious agents and host proteins. Starting from biochemical and biophysical approaches, such as SPR and microarrays in various flavors, she discusses several mass spectrometry approaches. She also covers briefly genetic screens using cDNA libraries and more extensively the newer RNAi-based methods, CRISPR/Cas9 technology, and genetic screens with haploid cells. A valuable addition for the discovery of microbe-host PPIs appears to be the genomic variation in the pathogen and host population exposed to it, revealing positive selection in several cases. Multiple examples are given for successful identification of pathogen receptors or pathogen-host interactions with components of the immune system using these newer technologies. She also describes the inherent difficulties when applying current high throughput technologies to this task due to the biochemical nature of membrane proteins, causing problems with solubility, correct folding, and posttranslational modifications. Also, the fast dissociation rates and low affinity for some of these interactions require further modifications not present in the natural proteins. Considering these issues, it becomes clear that high throughput approaches based on soluble PPIs are an excellent screening platform but results must be confirmed by thorough specific experiments in relevant cells and/or animals.

One family of secreted proteins that plays key roles in virus-host interaction is that of cytokines, essential to coordinate the innate and adaptive immune responses. Due to their function, it is not surprising to find that infectious agents have devised many strategies to modulate their activities. These strategies include the expression of proteins with the ability to bind cytokines and/or modulate their interaction with the cytokine receptor, as well as the expression of

transmembrane receptor and cytokine homologs. Among the cytokines, the interferons (IFN) are essential in protecting cells from viruses through the induction of an antiviral status. There are three types of IFN discovered to date: the multigene cytokine family of type I IFN containing IFN-α and β as prototypes, type II IFN including only IFN-γ, and type III IFN containing IFN-λ1–4. Two manuscripts deal with the interaction between IFN and virus infection in this special issue.

In the original research manuscript contributed by B. Hernáez et al., the authors study the effect of vaccinia virus (VACV) and its secreted, IFN-binding protein, B18, on the cellular type I IFN response. VACV is a member of the Poxviridae, large double stranded DNA viruses that encode many immunomodulatory proteins. In particular, they express secreted proteins with the ability to bind type I–III IFN [1–3]. VACV is one of the best-studied poxviruses in part due to its use as the vaccine employed during the world health organization-led campaign to eradicate smallpox, caused by the deadly variola virus. VACV expresses several genes whose protein products interfere with the IFN pathway and therefore can be used as a virus model to study evasion of IFN response. One of them, B18, is a secreted protein that binds type I IFN of different species with very high affinity, blocking the interaction between IFN and its receptor. Lack of B18 expression in VACV results in virus attenuation, indicating that it is a virulence factor [1]. By using a systems biology approach, the authors address whether B18 alone or in the context of VACV infection triggers changes in the global cellular transcriptome. They clearly show that the VACV B18 IFN-binding protein alone does not generally affect cellular gene expression and that replication competent VACV induces a global change in gene expression without enhancing that of IFN-related pathways. Similarly, they address whether addition of IFN has an effect on gene expression in the presence of VACV. Finally, they prove that VACV infection abrogated IFN-mediated antiviral response through both B18-dependent and independent mechanisms.

A different view on IFN is provided in the paper by J. Bruening et al. that reviews the current knowledge on IFN-λ expression and activity and its role during viral infection, focusing on hepatitis C virus (HCV). The authors describe the discovery and properties of IFN-λ and its receptor. IFN-λ signaling and induction of IFN-stimulated genes are, with some exceptions, quite similar to those of type I IFN. However, there are differences in the kinetics of activity and in the type of responsive tissue since expression of the IFN-λ receptor is restricted to epithelial cells. Most infectious agents, in particular viruses, infect epithelial cells at one or more stages of their life cycle. Therefore, the interaction between IFN-λ and pathogens may influence primary infection, spread, and pathogenicity. Focusing on the hepatotropic flavivirus HCV that can cause chronic infections leading to liver cirrhosis and hepatocellular carcinoma associated with high mortality, the authors describe HCV biology, pathogenesis, and current treatments. Newly developed antivirals targeting nonstructural proteins are highly successful in clearing HCV infection. However, due to their high cost and the fact that these do not inhibit reinfection, novel approaches are required. Finally, the authors review the innate immune response to HCV focusing on in vitro and in vivo studies addressing HCV-IFN-λ interaction. The authors discuss the interesting and initially counterintuitive data indicating that high levels of IFN-λ correlate with HCV chronicity of infection. Also, they explain the relationship between IFN-λ polymorphism, HCV spontaneous clearance, and the response to anti-HCV treatment.

The review article by J. M. Rojas et al. focuses on the anti-inflammatory master regulator IL-10 and its implications during viral infections. The main function of IL-10 is to limit immunopathology during inflammation, thereby protecting the host from tissue damage and loss of organ function. IL-10 is not only expressed by a variety of immune cells but is also able to regulate the activity of immune cells. It is, therefore, not surprising that viruses exploit this central immune regulatory pathway to evade immunity, leading to chronic/latent infections, and that detailed knowledge of the IL-10 effects is of high relevance. In this review, J. M. Rojas et al. discuss how the spatial and temporal delivery of IL-10 influences the course of viral infections. The focus of this review is factors that drive IL-10 expression during antiviral immune responses, cellular sources of IL-10, and IL-10-dependent regulatory mechanisms. These dynamic variables eventually impact how viruses can use this central regulatory pathway to undermine antiviral immune responses. The authors also discuss how a better understanding of both the basic expression level of IL-10 and effects of IL-10 on individual components of the immune system will enable the development of powerful immunotherapies against viral infections.

In another manuscript, K. Spiess et al. investigate a novel approach to treat human cytomegalovirus (HCMV), a highly prevalent beta herpesvirus that normally causes a primary asymptomatic infection. However, it causes disease associated with high morbidity and mortality in immunocompromised individuals, such as transplant recipients, and in the neonate. In this regard, HCMV is the leading infectious cause of congenital defects, above other pathogens like Zika virus. Therefore, development of novel therapeutic approaches to target HCMV is a public health priority worldwide.

In this study, K. Spiess et al. describe a therapeutic antiviral strategy against HCMV using immunotoxins, a protein-based therapy that takes advantage of known, specific ligand-receptor interactions. A typical immunotoxin contains a toxin linked to a targeting moiety to direct it to cells expressing the target receptor. Following binding to the receptor, the immunotoxin-receptor complex is internalized and the cell dies due to the action of the toxin. Several immunotoxins are currently being tested in clinical trials [4, 5]. The authors are pioneers in this field since they developed the first antiviral immunotoxin targeting the chemokine receptor US28 expressed by HCMV [6]. US28 is expressed at the plasma membrane of HCMV-infected cells and acts as a chemokine sink, binding and internalizing the ligands. Among the chemokines bound by US28 is CX3CL1, a chemokine that only interacts with one human chemokine receptor, CX3CR1. In this original research paper, the authors focus on CX3CL1-FTP, an immunotoxin containing a mutated form of CX3CL1 that specifically binds US28 over its cognate receptor CX3CR1 and that is linked

to the catalytic domain of *Pseudomonas* endotoxin A. Based on the property that US28 binds to CX3CL1 with higher affinity than other chemokines and is internalized upon interaction [7], they explore novel strategies as attempts to systematically improve the selectivity and antiviral activity of this immunotoxin. This immunotoxin specifically kills HCMV-infected cells being a promising anti-HCMV tool. Finally, they discuss the possibility of using similar strategies to kill cells infected with the oncogenic herpesviruses, Kaposi's sarcoma-associated herpesvirus, and Epstein–Barr virus.

In conclusion, this special issue highlights different recent advances in immunomodulation mediated by infectious agents, focusing on novel regulatory strategies that implicate secreted factors or their receptors of both host and pathogen origin. This compilation of papers thereby gives an overview over novel technologies and discoveries to study EPs, which regulate a variety of biological processes and represent therapeutic targets of emerging importance.

Disclosure

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Abel Viejo-Borbolla
Frank A. Schildberg
Hans-Gerhard Burgert

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Review Article

Technologies for Proteome-Wide Discovery of Extracellular Host-Pathogen Interactions

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Pathogens have evolved unique mechanisms to breach the cell surface barrier and manipulate the host immune response to establish a productive infection. Proteins exposed to the extracellular environment, both cell surface-expressed receptors and secreted proteins, are essential targets for initial invasion and play key roles in pathogen recognition and subsequent immunoregulatory processes. The identification of the host and pathogen extracellular molecules and their interaction networks is fundamental to understanding tissue tropism and pathogenesis and to inform the development of therapeutic strategies. Nevertheless, the characterization of the proteins that function in the host-pathogen interface has been challenging, largely due to the technical challenges associated with detection of extracellular protein interactions. This review discusses available technologies for the high throughput study of extracellular protein interactions between pathogens and their hosts, with a focus on mammalian viruses and bacteria. Emerging work illustrates a rich landscape for extracellular host-pathogen interaction and points towards the evolution of multifunctional pathogen-encoded proteins. Further development and application of technologies for genome-wide identification of extracellular protein interactions will be important in deciphering functional host-pathogen interaction networks, laying the foundation for development of novel therapeutics.

1. Introduction

The plasma membrane constitutes a critical biological interface between the cytosol and the extracellular environment of the cell, and consequently membrane-tethered proteins and secreted molecules (collectively termed extracellular proteins) are essential regulators of cellular communication. From high affinity cytokine-receptor interactions to low affinity cell-cell adhesion contacts, extracellular protein-protein interactions (ePPIs) are key for information processing and coordination of virtually all processes in a living organism. Furthermore, given their fundamental functions and their accessibility to systemically delivered drugs, extracellular proteins are particularly suitable targets for therapeutic intervention. In fact, despite these proteins being encoded by approximately one-fourth of the human genes, at least two-thirds of the existing drugs target either secreted or membrane-bound proteins [1]. Thus, the elucidation of the ePPI networks on a global scale has become crucial for the biomedical research. However, in spite of their relevance and abundance, ePPIs are remarkably underrepresented in available large-scale datasets. This discrepancy is due to the low sensitivity and limited compatibility of current high throughput technologies to detect extracellular interactions because of the unusual biochemical nature of the membrane proteins and the intractability of their binding partners [2–4]. In particular, transmembrane domain-containing proteins are amphipathic, making it difficult to solubilize them in their native conformation, and often contain posttranslational modifications such as glycans and disulfide bonds, which are not properly added in common heterologous expression systems [5]. In addition, interactions between cell surface proteins are often characterized by fast dissociation rates and therefore weak binding affinities, and in consequence well-established PPI methods such as yeast-two-hybrid or affinity purification-mass spectrometry (AP/MS) largely fail to detect these interactions. Over the last decade, several innovative technologies have been developed to overcome the aforementioned technical challenges and allow for sensitive

detection of ePPIs [2, 6–10]. Nevertheless, the mapping of ePPIs remains a major challenge in biology.

Infectious diseases result in millions of deaths each year and therefore identifying new candidate targets for improved therapeutic development remains a pressing health concern. Pathogens have evolved a myriad of elegant and often complex strategies to invade the host and commandeer host immune responses to allow pathogen replication, spread, and persistence in the infected organism. Many cell surface molecules serve as entry receptors for initial host cell invasion, and concerted responses to the pathogenic challenge critically rely on cell functions mediated by receptors and secreted proteins. To allow host colonization, pathogens encode highly optimized protein modulators, in the form of secreted molecules or receptors expressed on the plasma membrane of the infected cells or the surface of the pathogen [11, 12]. Interactions between these proteins and extracellular host molecules form the foundation of communication between a host and a pathogen and play a vital role in the initiation and outcome of the infection [13, 14]. Characterizing host-pathogen ePPI networks is therefore of utmost importance to gain a better understanding of the infection process and to inform the development of novel or improved therapeutic strategies. Excellent studies on mapping host-pathogen interactions, particularly MS-based analysis of viral infection, have provided a wealth of insight into infectious diseases [15–19]. Nevertheless, similarly to host ePPIs, a significant hurdle to the elucidation of host-pathogen biology has been the shortage of datasets of extracellular interactions between host and pathogen proteins, partly due to the technical challenges that these proteins present. Moreover, an additional consideration when studying pathogen-encoded molecules is that these proteins often lack any recognizable homology with any host molecules, thus precluding prediction of their functions [11, 20]. Robust methodologies that permit unbiased characterization of ePPI in the absence of preexisting hypotheses are thus needed to elucidate the binding partners and molecular functions of most pathogen-encoded molecules.

Excellent reviews have recently revisited the currently available technologies for proteome-wide ePPI discovery [4, 8, 53–55]. Here we discuss the application of some of these technologies to the study of host-pathogen interaction and describe some of the major findings that have recently impacted research in the field of extracellular host-pathogen recognition. Protein microarrays and functional genomics approaches are highlighted here as emerging techniques with unique potential for the elucidation of host-pathogen ePPI networks at a genome-wide scale.

2. Biochemical and Biophysical Approaches

Classical biochemical and biophysical approaches are particularly suitable for detection of high affinity host-pathogen interactions, such as those mediated by a viral capsid protein and a host cell surface receptor, or a pathogen-encoded glycoprotein and a secreted host factor. Typically, these approaches have relied on the utilization of recombinant pathogen proteins as baits to probe for host binding partners, followed by immunoprecipitation and MS, or biophysical techniques for analysis of PPI such as surface plasmon resonance (SPR). SPR requires prior knowledge of the possible interacting partners and is therefore unsuitable for unbiased PPI discovery, whereas immunoprecipitation and MS approaches usually fail to detect weak interactions, which often characterize ePPIs, particularly those that take place on the cell surface. Notwithstanding, the identification of the receptors for some of the most prominent pathogens, such as the severe acute respiratory syndrome coronavirus (SARS-CoV) or the New World Arenaviruses, was made utilizing standard immunoprecipitation techniques [56, 57]. Despite their inherent limitations, biochemical approaches continue to provide relevant insights into host-pathogen interactions, such as the discovery of the dipeptidyl peptidase 4 (DPP4) as the receptor for the MERS-CoV just within a few months after the emergence of this virus [58]. In addition, more recently Kabanova and colleagues identified the cell surface receptor for the trimeric entry complex gHgLgO encoded by human cytomegalovirus (hCMV) [21]. Several studies have shown that the gHgLgO trimer is involved in the infection of fibroblasts, whereas the gHgLUL128L pentameric complex is required for entry into endothelial, epithelial, and myeloid cells [59–61]. In this work, both the trimeric and the pentameric CMV protein complexes were generated as recombinant products and used as baits to perform binding experiments on biotinylated cell surfaces, followed by immunoprecipitation and MS identification of bait-cell receptor complexes. Using this approach, the authors identified the cell surface protein PDGFRα as a high affinity receptor for the gHgLgO trimer and demonstrated that this interaction was required for infection of fibroblasts. Interestingly, in the case of the pentameric CMV complex, multiple bands were detected upon protein immunoprecipitation from epithelial cells, suggesting the existence of multiple receptors on these cells, which so far remain unknown [21] (Table 1).

Different biophysical techniques for detection of PPI, in particular SPR, have also proven valuable in the field of host-pathogen interactions. SPR offers the advantage of label-free, sensitive detection of interactions between a diversity of ligands in real time, thus allowing calculation of kinetic parameters. SPR has been widely utilized to monitor antibody binding to a variety of pathogen antigens, information that has informed vaccine development [62–64]. The SPR technology has also been exploited for discovery of ePPIs. For example, Viejo-Borbolla and coworkers utilized SPR to screen several secreted and membrane-expressed glycoproteins encoded by herpes simplex viruses (HSVs) for binding to chemoattractant cytokines (the chemokine family) and were able to identify a subset of human chemokines that bound to HSV glycoprotein G with high affinity [22]. Recently, Day and colleagues utilized a combination of glycan arrays and SPR and identified over 60 host-bacterial glycan pairs characterized by a wide range of binding affinities, some of which participated in bacterial adherence to host cells in vitro, leading to the hypothesis that bacteria-host surface glycan interactions may mediate initial attachment to the target cell during infection [65]. Despite SPR and related methods offering higher sensitivity for detection of transient

Table 1: Overview of the main high throughput approaches utilized to detect ePPI between pathogens and their respective hosts and relevant examples discussed thorough the text.

Pathogen	Identification method	Main findings	References
Human cytomegalovirus (hCMV)	Biochemical and MS	PDGFRα identified as a high affinity cell surface receptor for the CMV gHgLgO protein complex	[21]
Herpes simplex viruses (HSVs)	Biophysical	Secreted and plasma membrane-expressed glycoprotein G targets a specific set of human chemokines with high affinity	[22]
Human immunodeficiency virus type 1 (HIV)	Monoclonal antibodies	CD4 identified as the receptor for HIV infection of T cells	[23, 24]
Rhinovirus	Monoclonal antibodies	ICAM-1 as the common entry receptor for most rhinovirus serotypes	[25, 26]
Plasmodium falciparum	AVEXIS	BASIGIN identified as the cell-surface receptor that mediates erythrocyte infection	[27]
Human adenoviruses	Extracellular human protein microarrays	Elucidation of the extracellular interactome of adenovirus-encoded immunomodulatory proteins	[28]
Pseudomonas aeruginosa	Extracellular pathogen protein microarrays (NAPPA)	Screening of patient sera against all *P. aeruginosa* extracellular proteins. 12 proteins identified as potent antigens	[29]
Varicella zoster virus (VZV)	Extracellular pathogen protein microarrays (NAPPA)	Identification of 18 extracellular viral proteins that promote humoral responses upon screening of the entire VZV proteome	[30]
Streptococcus	Extracellular pathogen protein microarrays	Identification of new streptococcal proteins that interact with fibronectin, fibrinogen, and C4BP factors	[31]
Hepatitis delta virus LHDAg antigen	Plasma membrane microarrays (MPA)	150 candidate interactions identified between viral antigen and plasma membrane proteins	[32]
Simian virus 40 (SV40)	Plasma membrane microarrays (MPA)	99 candidate interactions between whole particles and plasma membrane proteins identified	[32]
Vaccinia virus (VACV)	TRICEPS (MS)	7 candidate cell surface binding partners identified for VACV	[33]
Viral pathogens	Computational studies	Insights into global principles of virus-host PPI networks	[15–18, 34–36]
Hepatitis C virus (HCV)	cDNA libraries	CD81, Claudin-1, and Occludin as cell surface receptors and some of the players involved in HSV internalization	[37–39]
Adenovirus and coxsackievirus B	Monoclonal antibodies and cDNA libraries	CAR identified as a common entry receptor for adenovirus 2/5 and coxsackievirus B	[40]
Sindbis virus	siRNA screens	NRAMP as cell surface receptor for entry into *Drosophila* cells	[41]
Murine norovirus	CRISPR/Cas9	CD300lf identified as a cell surface receptor that determines virus tropism	[42]
Bacterial distending toxins *(E. coli)*	Haploid cell screens	Sphingomyelin synthase 1 and the putative G protein-coupled receptor TMEM181 identified as toxin receptors	[43, 44]
Clostridium difficile and *perfringens* toxins	Haploid cell screens	Lipolysis-stimulated lipoprotein and the low-density lipoprotein receptor-related protein 1 identified as the receptors for the bacterial toxins, respectively	[45, 46]
Ebola virus	Haploid cell screens	HOPS proteins and the Niemann-Pick C1 (NCP1) transporter identified as endosomal receptors that mediate cytosol access	[47]
Lassa virus	Haploid cell screens	LAMP1, lysosomal-associated membrane protein 1 identified as an essential host factor mediating virus release to cytosol	[48]
Adeno-associated virus (AAV) serotype 2	Haploid cell screens	46 cell host factors identified, including heparin sulfate proteoglycan biosynthesis and intracellular transport genes. The immunoglobulin domain-containing transmembrane protein KIAA0319L identified as AAV receptor	[49]

Table 1: Continued.

Pathogen	Identification method	Main findings	References
Plasmodium falciparum	Population genomics analysis	*P. falciparum* EBL-1 protein binds to the erythrocyte receptor glycophorin B, a highly polymorphic gene in malaria-endemic regions	[50]
Streptococcus	Phage display	19 bacterial proteins identified as potential fibronectin-binding proteins	[51]
Fusobacterium nucleatum	Transposon-based mutant libraries	The bacterial protein Fap2 binds to the receptor TIGIT and downregulates NK-mediated killing of tumor cells	[52]

AVEXIS, Avidity-based extracellular interaction screen; NAPPA, nucleic-acid programmable protein array; MPA, microfluidic-based comprehensive protein array; CRISPR, clustered regularly interspaced short palindromic repeat; MS, mass spectrometry; PPI, protein-protein interaction.

PPIs than most biochemical approaches, these biophysical techniques have not been exploited for large-scale ePPI discovery, possibly due to the low throughput of the available instrumentation and the overall difficulties for generation of the relevant protein libraries.

These studies, among many others, have demonstrated the power of the classical biochemical and biophysical techniques for detection of host-pathogen interactions. Nevertheless, these approaches require previous knowledge of the pathogen-encoded proteins responsible for binding and the ability to produce such proteins as recombinant reagents, which may be challenging, as exemplified by the production of hCMV entry complexes [21, 66]. Alternative methods have been utilized in those cases where there is no previous knowledge of the pathogen proteins required for interaction with the host cells. In this regard, the screening of large collections of monoclonal antibodies raised against membrane proteins has proven particularly useful to identify receptors that mediate viral entry. Back in the early 80s, the discovery of CD4 as an entry receptor for the human immunodeficiency virus type 1 (HIV) significantly impacted our understanding of viral pathogenesis and subsequent development of therapeutics [23, 24]. In this case, the well-defined tropism of the virus determined the choice of over 100 antibodies directed against human leukocyte differentiation antigens, of which only antibodies that recognized the surface receptor CD4 blocked viral infection [23]. It is worth noting that similar monoclonal antibody screens have also been utilized for unbiased characterization of viral blockers. For example, Colonno and colleagues performed a screen of more than 2,000 hybridomas from mice immunized with preparations of plasma membranes from human cells and were able to find one antibody that blocked rhinovirus binding to its cell surface receptor [25], identified as the intercellular cell adhesion molecule 1 (ICAM-1) in subsequent studies [26].

Despite the undoubted importance of the biochemical and biophysical approaches to the study of host-pathogen interactions, the aforementioned limitations have motivated the development of alternative technologies for large-scale analysis of ePPIs.

3. Protein Microarrays

From the initial utilization of microarrays for detection of PPI over a decade ago, human proteome chips containing thousands of recombinant proteins have been generated, some of which are now commercially available. Protein microarrays offer the unique advantage of requiring minimal consumption of protein reagents, fast readouts, and relatively more affordable instrumentation. Typically, a fluorescently labeled or tagged protein of interest (the bait) is generated as a recombinant product and screened against all proteins in the array [10, 53]. Despite the increased availability of high-coverage protein arrays, very few are focused on extracellular proteins and therefore are not suitable for study of host-pathogen ePPIs. Most existing microarray-based methodologies rely on multimerization of the bait protein for increased avidity and detection of weak ePPIs, mimicking the way these interactions occur in vivo, where proteins are arrayed in the crowded molecular environment of apposing plasma membranes. Different multimerization strategies and protein microarray libraries have been developed and utilized for host-pathogen interaction discovery, some of which are described in more detail below.

3.1. Avidity-Based Extracellular Interaction Screen (AVEXIS). The Wright lab developed a novel method for detection of low affinity ePPIs, termed avidity-based extracellular interaction screen (AVEXIS) [2]. In brief, AVEXIS consists of the expression of the extracellular domain (ECD) of the bait of interest as a recombinant protein, which retains its binding properties while removing the insoluble transmembrane region of the protein. These ECDs are tagged with a coiled-coil sequence from the rat cartilage oligomeric matrix protein to allow for pentamerization of the bait and therefore increased binding avidity, alongside a β-lactamase tag for detection of bait-prey interactions upon incubation with the colorimetric substrate nitrocefin. This multivalent strategy has been used for medium-scale screens, allowing detection of weak interactions between human receptors with low false-positive rates [2]. Notably, Crosnier and colleagues applied AVEXIS to search for the plasma membrane receptor responsible for *Plasmodium falciparum* infection of erythrocytes [27]. The authors compiled a library consisting of most secreted or cell surface-expressed proteins in erythrocytes and systematically assayed more than 40 red blood cell proteins for binding to *P. falciparum* protein PfRh5, a parasite protein essential for blood stage growth, expressed as an AVEXIS pentameric bait. Notably, the Ok blood group antigen BASIGIN was identified as a unique receptor for PfRh5, and inhibition of this interaction was shown to be sufficient to block parasite invasion of the erythrocyte, findings that may importantly inform antimalarial therapies [27]. In later studies, AVEXIS was miniaturized making this approach compatible with the protein microarray format, thus permitting more comprehensive and lower resource-intensive screenings [67]. Although this technique should allow for high throughout and sensitive determination of ePPIs, this approach has not yet been applied to elucidation of pathogen-host interactions.

3.2. Extracellular Protein Microarray Platforms. Over a decade ago, fueled by the recent completion of the human genome, Genentech pioneered a significant effort to identify novel secreted or transmembrane domain-containing proteins, upon careful bioinformatics assessment and high throughput protein purification [68]. These efforts resulted in the generation of a comprehensive human protein library, which was subsequently utilized to develop an extracellular protein microarray platform, consisting of over 1,500 secreted or single-transmembrane domain containing proteins [10]. For the generation of this human protein library, secreted proteins or the ECD of single-transmembrane receptors were fused to different affinity tags and subsequently purified from cell culture supernatants by size-exclusion chromatography. Mammalian cells or baculovirus-insect cells were preferentially used as expression systems, to maximize the likelihood of proper folding and glycosylation of the extracellular protein collection [10, 69]. SDS-PAGE and multiangle laser

light scattering were used to analyze noncovalent aggregation and ensure high-quality protein production. Subsequently, the purified proteins were spotted on epoxysilane slides using a NanoPrint Arrayer, and protein immobilization on the microarray was determined by probing the slides with the relevant anti-tag antibodies [10]. To enhance detection of low affinity interactions, a rapid method to assemble bait proteins (whose ECD was expressed as a Fc tag-fusion protein) into multivalent complexes using fluorescently labeled protein A microbeads was developed. Proof-of-concept assays showed high sensitivity for detection of weak ePPIs characterized by micromolar K_D, a minimal off-target binding, and more than 70% true-positive to false-positive detection ratio [10, 69]. Over the years, this extracellular protein microarray has successfully identified counterreceptors for a number of human molecules, providing relevant insights into novel pathways that coordinate a multitude of cell functions [70–72].

3.3. Protein Microarrays for Viral Immunomodulatory Protein Receptor Discovery. Recently, we applied this ePPI discovery platform to the study of extracellular viral proteins (Figure 1), with a focus on human adenovirus- (HAdV-) encoded immunomodulatory proteins [28]. Despite the increasing relevance of HAdV as both pathogens and therapeutic vectors, information on the interaction of these viruses with the host immune system remains scarce [73, 74]. Interestingly, the immunomodulatory proteins encoded by these viruses, termed E3 proteins, show substantial diversity in their ECDs across and within viral species and constitute one of the most divergent regions of the HAdV genome [75, 76]. Given this striking variability, the E3 proteins have been suggested to play a role in viral tropism and pathogenesis, yet the functions of virtually all E3 proteins have remained unknown [73]. In our study, we took advantage of such unique variability to evaluate the effect of viral immunomodulatory protein diversity in extracellular host targeting. Screening of a substantial number of E3 proteins encoded by different HAdV species using the extracellular protein microarray platform allowed identification of over 50 novel virus-host interactions encompassing 5 viral species, which were fully validated by orthogonal methods [28]. These findings revealed significant diversity in extracellular host targeting and, moreover, allowed identification of semiconserved host targets, pointing towards specific human receptors that may represent previously unrecognized hubs for viral perturbation. Furthermore, most of the E3 immunomodulators were identified as multifunctional proteins, suggesting that viruses have evolved proteins capable of interfering with several cellular functions, a strategy consistent with the optimization of limited genomic resources. Such economic targeting has been often observed in intracellular targeting [15–17], but so far few examples of widespread targeting in the extracellular environment have been reported [28, 77–80], let alone a global elucidation of ePPI networks, in part due to the technical challenges associated with ePPI detection.

Remarkably, many of the HAdV E3 proteins preferentially interacted with host receptors that exert known or predicted inhibitory functions during the immune response (as defined by the presence of intracellular immunoreceptor tyrosine-based inhibitory motif, ITIM), including LILRB1 [81, 82], LAIR1 [83], and MPZL1 [84], suggesting previously unrecognized strategies of immunosuppression that may be utilized by other human pathogens [28]. Moreover, several of the receptors identified as targets for the viral proteins in this study (including the prominent cell surface molecule CD45) do not have known counterreceptors in the host, supporting the longstanding hypothesis that pathogen molecules drive the evolution of immune receptors and in many instances may represent the most relevant modulators of host receptor function [85–87]. In summary, such unbiased, microarray-based study of immunomodulatory proteins represented the first large-scale analysis of the PPI landscape of a collection of extracellular immunomodulators encoded by viruses. Future investigation of other pathogen-encoded molecules using similar extracellular protein microarrays will likely shape our understanding of the pathogen imprint in our immune system.

3.4. In Vitro Transcription and Translation- (IVTT-) Based Microarrays. One of the main limitations of any protein microarray platform is the lower protein coverage relative to other genome-wide methods for PPI identification, due to the costs and difficulties for generation of comprehensive protein libraries to be deposited onto the microarrays. In an attempt to address this caveat, Ramachandran and colleagues developed a method called nucleic-acid programmable protein array (NAPPA), in which DNAs are directly deposited onto the array followed by protein synthesis in situ using an in vitro transcription and translation (IVTT) system, thus avoiding the need for protein purification [88]. Although this promising approach has proven superior in generating transmembrane-containing molecules as soluble proteins, it still remains to be systematically addressed if the extracellular human proteins produced in this manner present the folding and posttranslational modifications necessary for protein functionality. Nevertheless, emerging data support the utility of NAPPA as a useful tool for the study of bacterial proteins. For example, Montor and colleagues used a bioinformatics approach to predict the *Pseudomonas aeruginosa* proteins that reside in the outer membrane of the bacteria or are secreted to the extracellular environment of the infected cell [29]. In this work, the authors utilized the NAPPA approach to screen all predicted extracellular gene products for interaction with sera from cystic fibrosis patients, where *P. aeruginosa* establish a life-threatening lung infection. From 266 bacterial proteins initially selected, 12 proteins were recognized by antibodies in the sera, indicating that these bacterial proteins represent major antigens that trigger adaptive immune responses in humans. Interestingly, robust antibody responses against three previously uncharacterized proteins were detected, suggesting this approach could help identify new extracellular proteins that exert unknown functions during the infection [29]. These results confirmed the utility of the microarrays to detect immune responses against membrane proteins encoded by pathogens, and supported the use of this methodology for diagnosis applications. In this regard, several groups have developed microarrays composed

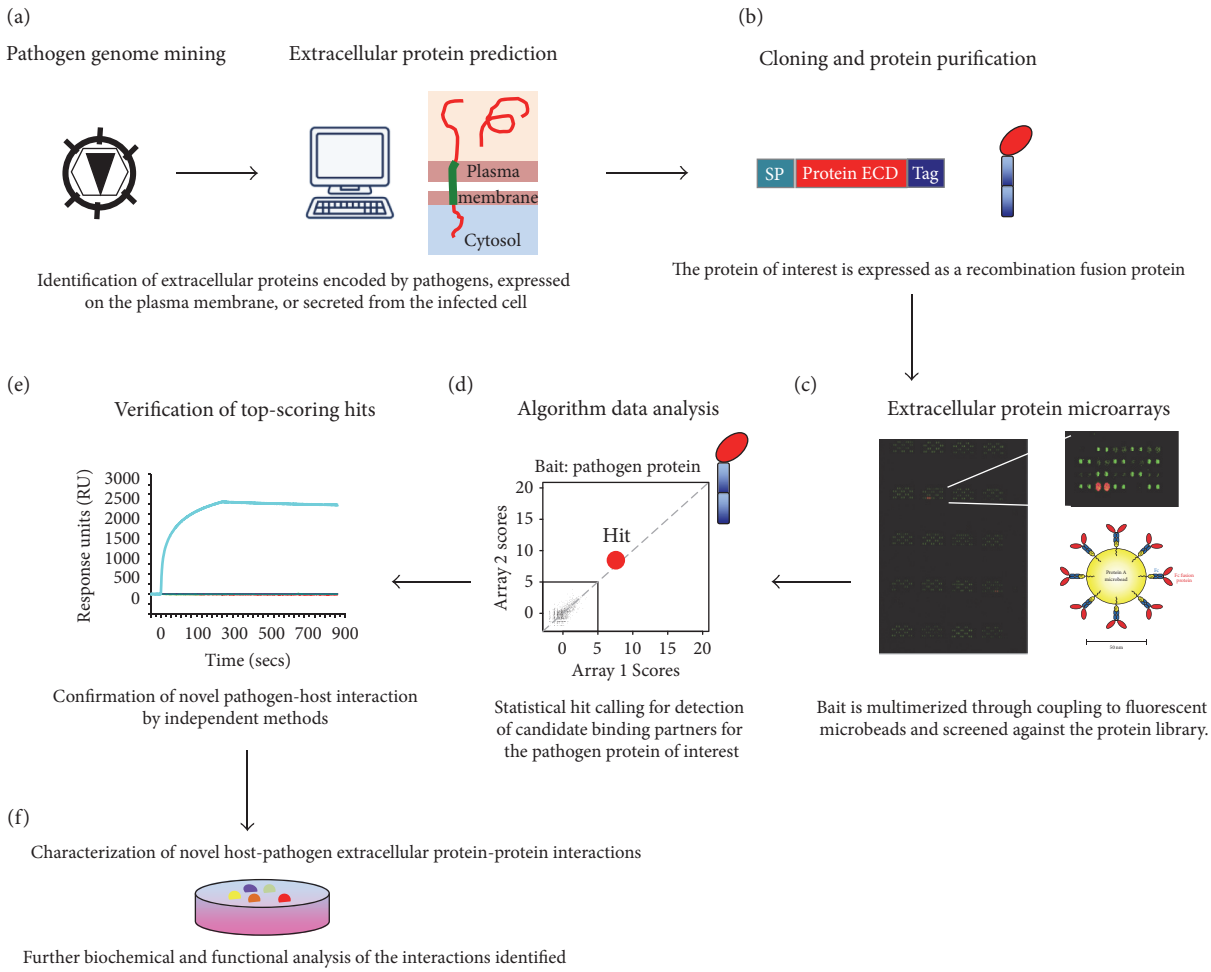

Figure 1: Overview of the application of the protein microarrays technology for extracellular pathogen-host protein-protein interaction discovery. (a) Identification of genes that encode for secreted factors or cell surface-expressed proteins, based on published data or bioinformatics analysis. (b) Cloning, expression, and purification of the pathogen-encoded proteins of interest. The full-length protein (secreted proteins) or the ECD (transmembrane-containing proteins) is fused to a tag for subsequent expression in the heterologous system of choice followed by affinity purification. Mammalian or baculovirus-based systems are preferred to allow for introduction of posttranslational modifications. (c) Screening of the selected pathogen-encoded proteins (baits) against extracellular human protein libraries using protein microarray technologies. Different strategies for bait multimerization have been developed to allow for detection of lower affinity interactions (see text for details). A multimerization strategy based on the coupling of Fc-tagged baits to fluorescent protein A microbeads is shown. Additional microarray-based technologies have been developed to avoid the need for extensive protein purification associated with library generation (see text for details). (d) Algorithm analysis of the protein microarray data. Frequent nonspecific binders in the human library are filtered out, and binding partners for the pathogen protein under study are depicted as high-scoring, intersecting hits. (e) Validation of the interaction between the pathogen-encoded protein of interest and the novel receptor(s) identified in the screens. Experimental validation of the protein-protein interactions may be performed using orthogonal approaches, such surface plasmon resonance, immunoprecipitation, and flow cytometry. (f) Selected binding partners may be further characterized biochemically and functionally to assess the relevance of the novel pathogen-host interactions identified. SP, signal peptide; ECD, extracellular domain.

of pathogen-encoded proteins [30, 89–93]. Such pathogen protein arrays have so far being exploited mainly for diagnosis purposes, to allow screening of antibodies present in patient sera for binding to extracellular bacterial or viral antigens on the array. Nevertheless, their inherent high throughput and compatibility with multivalent bait approaches makes them a powerful tool for ePPI discovery. For example, Margarit et al. developed a *Streptococcus* microarray to find novel microbial proteins capable of binding to the human proteins fibronectin, fibrinogen, and C4BP and were able to identify a set of streptococcal proteins that interacted with these factors [31]. Nevertheless, despite such pathogen protein-based arrays offering great promise, this methodology remains to be systematically analyzed for ePPI discovery.

More recently, Yu and colleagues applied NAPPA technology in combination with the HaloTag-Halo ligand detection system to elucidate the interaction network of two effector proteins (SidM and LidA) encoded by *Legionella pneumophila*, a highly pathogenic bacteria that is the causative agent of Legionnaire's pneumonia [94]. Similarly to many

pathogen proteins, these virulence factors lack significant homology to host molecules therefore complicating the assessment of their host targets and biological functions. In this work, the bacterial proteins of interest were tagged with a HaloTag, a modified haloalkane dehalogenase that covalently binds to synthetic Halo-ligands (haloalkanes) that can be fluorescently labeled, thus allowing more robust detection of bait protein binding to interactors present on the array. In this study, more than 10,000 human proteins were expressed on the NAPPA array using different IVTT techniques, leading to identification of 20 and 18 binding partners for the LidA and SidM effectors, respectively, most of them experimentally verified by pull-down [94]. Although this study focused on identification of intracellular PPI, the applicability of the NAPPA-HaloTag technology for ePPI determination should be explored in the future. Moreover, bait multimerization strategies should be implemented in order to make this approach more suitable for detection of transient PPIs.

Related to the NAPPA technology, Glick and colleagues recently built a miniaturized platform focused on human membrane proteins. By integrating the microfluidics technology, protein microarrays, and an IVTT system, this group built a new device named microfluidic-based comprehensive human membrane protein array (MPA) [32]. A notable improvement introduced by these investigators was the addition of microsomal membranes to the IVTT system to allow for improved folding and posttranslational modifications in plasma membrane proteins, both common limitations of IVTT systems. In this work, a library of 2,700 human genes encoding for membrane proteins was built and subsequently utilized to screen the large-form delta antigen (L-HDAg) encoded by the hepatitis delta virus (HDA) and whole viral particles of the simian virus 40 (SV40), a nonenveloped human pathogen. Proof-of-concept assays showed encouraging results, with over 75% true-positive rate within a small set of proteins with known interactors and, more importantly, indicated the feasibility of this approach for expression of multitransmembrane-containing proteins, a protein type that has proven challenging given their high hydrophobicity. The MPA screens identified 99 and over 150 interactions for SV40 particles and L-HDAg viral protein, respectively, and around 35 interactions were validated by coimmunoprecipitation or protein-fragment complementation assays [32]. To our knowledge, this is the first study to assess ePPIs using a comprehensive human protein library and whole viral particles (SV40) as baits, a valuable approach that may provide important insights into pathogen tropism, alongside a molecular explanation for the cell surface receptors engaged by the pathogen. Further utilization of this platform followed by a more systematic analysis of the candidate hits, including nonspecific binder determination, will be needed to assess the overall performance of the MPA technology. Regardless, this platform provides an extended version of the NAPPA approach that focuses on mammalian ePPIs and may therefore provide relevant insights into extracellular host-pathogen interactions.

The protein microarrays have represented one of the most fruitful approaches for unbiased determination of ePPIs, including host-pathogen interactions. Nevertheless, one of the main limitations of this technology is the need to generate comprehensive libraries, a process that is resource consuming and often not available to many researchers [53, 93]. Consequently, although some of the available arrays were designed to cover a significant fraction of the human proteome, any discoveries made using these platforms are limited to the proteins present in each array. The current libraries are likely to continue expanding alongside innovative approaches to facilitate sensitive detection of ePPI using protein microarray formats.

4. Mass Spectrometry-Based and Computational Approaches

Over the last decade, MS-based technologies have emerged as a versatile, powerful approach to decipher many aspects of the human proteome, including the characterization of protein complexes. Excellent reviews on current MS-based technologies, recent improvements, and future prospects for elucidation of PPI networks are available [95–98]. In this review, we briefly discuss the applications of some of these techniques to the study of extracellular host-pathogen interactions.

4.1. Mass Spectrometry-Based Characterization of Membrane Proteins and Interacting Partners. The proteins expressed on the surface of pathogens mediate functions necessary for survival, replication, immunoevasion, and transmission and therefore are logical candidates for therapeutic and vaccine design. However, the study of the surface proteome in pathogens, particularly in bacteria is constrained by the fact that commonly used prediction algorithms fail to correctly predict the location of several proteins [29, 99–101]. Despite the characterization of the extracellular proteins and their interactions still representing the Achilles heel of most proteomics methods, MS has emerged as an invaluable approach to characterize the protein composition of plasma membranes [5]. To date, several studies have exploited MS-based techniques to gain insights into the extracellular protein composition of bacterial pathogens [101–104]. For example, Palmer and colleagues studied the surface proteome of the tick-borne intracellular pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) using liquid chromatography and tandem MS [105]. Interestingly, the authors found that the surface proteome of *A. marginale* isolated from tick cells, despite being less complex than that of bacteria isolated from human erythrocytes, contained a novel protein, which the authors hypothesized to play a function in human cell invasion in spite of its human counterreceptor remaining uncharacterized. This interesting observation suggests a remodeling of the bacteria surface proteome during the transition between mammalian and arthropod hosts, an aspect of the infection that could be targeted to block transmission. Similarly, several studies have pursued the identification of the proteins present in viral particles utilizing MS. Although these analyses suffer from several drawbacks associated with membrane protein characterization, particularly the poor solubility of these proteins and the low abundance of many plasma membrane

proteins, these studies have revealed a complex composition for most of the viruses studied, alongside incorporation of many host proteins in the virions, in most cases with undetermined functions [106–108].

An interesting observation from some of the studies referred above is the fact that certain bacterial proteins, predicted cytoplasmic by consensus, can be found in the extracellular environment of the cell, where they may play alternative functions. In fact, the number of proteins that are secreted through noncanonical signal sequence pathways is increasingly appreciated [99, 100, 109, 110]. Little is known about these bacterial proteins originally described as cytosolic proteins but capable of exerting functions on the cell surface, which some authors have named moonlight proteins, in reference to their potential to exert multiple functions [111]. There is emerging evidence that protein moonlighting contributes to virulence of important bacterial pathogens including *Staphylococcus aureus* or *Mycobacterium tuberculosis*, sometimes in fascinating ways. For example, *M. tuberculosis* is known to encode two molecular chaperones, Cpn60.1 and Cpn60.2, which function as modulators of myeloid cells among other regulatory functions [112]. Despite these chaperones being by definition cytosolic, Cnp60.2 has been detected in significant amounts on the bacterial surface, and either recombinant Cnp60.2 or antibodies against this protein efficiently block binding of *M. tuberculosis* to macrophages [112], through a potential interaction with the receptor CD43 [113]. In addition, the protein DnaK, a Hsp70-related protein encoded by *M. tuberculosis*, can locate to the bacterial surface and functionally interact with CD40 [114] and with the HIV coreceptor CCR5 [115]. Notably, DnaK appears to block HIV binding to CCR5 in vitro, an interesting observation given the co-occurrence of *M. tuberculosis* and HIV infection [116]. Although a more detailed revision is out of the scope of this review, bacterial protein moonlighting, excellently revisited by Henderson and Martin [111], is a thought-provoking phenomenon that suggests a much more complex extracellular landscape than anticipated. Moreover, such protein moonlighting is in line with the hypothesis that pathogens have evolved multifunctional proteins as a prominent strategy for efficient use of limited genomic resources [17, 28].

Another MS-based approach that holds great promise for host-pathogen ePPI detection is the recently developed TRICEPS [33]. TRICEPS is a chemoproteomic reagent that consists of three moieties, one that binds the ligand of interest through its amino groups, a second one that binds glycosylated receptors on the cell surface, and a biotin tag for purifying the receptor peptides for subsequent identification by MS. Notably, in the initial description of the method, TRICEPS was successfully applied to the identification of receptors for extracellular ligands of diverse nature, such as secreted glycoproteins, small peptide ligands for G protein-coupled receptors, and therapeutic antibodies. Importantly, this approach has also been utilized to study cell surface molecules targeted by vaccinia virus (VACV). Interestingly, the analysis of VACV binding to HeLa cells revealed seven candidate binding partners, including the previously identified receptors AXL, chondroitin sulfate proteoglycan 4, and laminin binding protein dystroglycan 1. Further, downregulation of five out of the seven candidates using short interfering RNA reduced VACV infection by 40–60%, supporting the functionality of the interactions identified, at least in vitro [33]. Although this technology is still developing and no studies on other pathogens have been published yet, future TRICEPS-based studies promise relevant insights into pathogen interaction with distinct components of the cell surface.

4.2. Computational Approaches for Characterization of Pathogen-Host Interactions. As an addendum to the vast amount of knowledge acquired using MS approaches and some of the additional methodologies discussed in this review, bioinformatics offers an in silico systems biology approach that reveals a global perspective on host-pathogen interactions. Advances in computation have been fundamental to dissect the complex datasets generated in many genome-wide MS-based studies and have enabled the reconstruction of large-scale host-pathogens PPI networks, providing fundamental insights into viral disease and hence host biology [15–18, 34–36, 117]. Although the computational tools available for analysis of large datasets, in some cases developed in association with some of the high throughput screens mentioned in this review, certainly deserve a focused chapter, a couple of observations are specially notable. Commonly observed in these studies is that the intracellular viral effectors preferentially target host proteins that act as hubs (proteins with many interacting partners) or bottlenecks (proteins central to many pathways in the network) [15, 16, 36]. For example, Dyer and colleagues built a network of host-pathogen PPI by integrating published information from 190 pathogens [36]. Supporting previous findings, this analysis indicated that pathogen-encoded proteins preferentially interfere with host molecules that control critical cellular processes, such as cell death or nuclear transportation, possibly as a strategy to maximize control of the host machinery given limited genomic resources. Interestingly, this study highlighted a small set of extracellular host proteins recurrently targeted by several of the viral and bacterial pathogens analyzed, including cell surface receptors such as VEGFR2/KDR and collagen, possibly indicating previously unrecognized roles in the immune response against pathogens. Although informative, the analysis performed by Dyer and collaborators was skewed towards viruses, with a prominent enrichment in HIV strains [36]. More extensive analyses encompassing other human viruses and bacterial pathogens may reveal general strategies of immunomodulation and potential human targets suitable to therapeutic intervention. Interestingly, increasing evidence suggests that virus-host interactions are governed by principles distinct to those that dictate within-host interactions [20, 28, 85, 87, 118]. Notably, detailed analyses carried out by the Xia group highlighted significant differences between virus-host and within-host (also called endogenous) interactions, such as the tendency of viral proteins to compete with host proteins for binding to a given receptor in the absence of sequence similarity with the host counterpart or the observation that viral molecules have evolved multiple short linear motifs capable of mediating a number of diverse interactions

[20, 118], features that are consistent with the multifunctional capabilities of some pathogen-encoded proteins [20, 22, 28, 77–80, 118]. Altogether, bioinformatics analysis of virus-host interactions suggest that virus-mediated targeting of host proteins is characterized by signatures of pleiotropy, economy, and convergent evolution, conclusions that are supported by emerging experimental data. Followed by thorough biological experimentation such computational-based systems biology approaches will provide a unique tool to help decipher basic global principles of pathogen-host interaction and may reveal novel ePPIs amenable to therapeutic intervention.

5. Genetic Screens

5.1. Complementary DNA Libraries and RNA-Interference-Based Approaches. Alongside protein microarrays-based technologies, MS, and computational analysis, the explosion of the functional genomics field in the last years has revolved the avenues to study pathogen interactions with their hosts, often in high throughput. In brief, genetic screens comprise gain-of-function and loss-of-function strategies, represented by complementary DNA (cDNA) libraries and RNA-interference- (RNAi-) based approaches, respectively. These methods were developed more than two decades ago and have been widely utilized by the scientific community, providing fundamental insights into the infection process. In particular, the cDNA libraries have proven extremely successful in identifying viral receptors through a gain-of-function approach, upon transduction of the cDNA library from a susceptible cell line into nonpermissive cell lines. The use of cDNA libraries is not reviewed in detail here in the interest of a more comprehensive revision of relative newer genomics-based approaches, such as the clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) or the haploid cell screens. Nevertheless, these libraries have represented one of the most significant technologies to further our understanding of the pathogen-host interaction. For example, early studies made use of cDNA libraries to shed light on the complex mechanism exploited by hepatitis C virus for initial invasion of the cell [37–39], identified CAR as a common receptor for adenovirus 5 and coxsackievirus B [40], and were instrumental to identify SLAM1 and PVR as a receptors for measles and poliovirus, respectively [119, 120].

In turn, the RNAi technology has yielded significant insights into virus-host interactions, such as the identification of the ion transporter NRAMP as the receptor for the mosquito-borne Sindbis virus colonization of *Drosophila* cells [41]. The main power of the RNAi technology is that it allows high throughput genome-wide screens and therefore potential identification of essential factors that play roles in different aspects of the pathogen life cycle, including initial interaction with the host cell. Although RNAi screens have provided tremendous insights into host-pathogen interactions and remain widely utilized [121], inefficient gene depletion and off-target effects are important limitations of this methodology [122].

5.2. CRISPR/Cas9-Based Screening Technology. The increasingly popular CRISPR/Cas9 technology overcomes some of the caveats often associated with genetic manipulation and holds enormous promise for genome editing and downstream applications, including host-pathogen interaction discovery [123]. Although still early days, high throughput CRISPR/Cas9 screens for genome-wide studies have already displayed remarkable results, with high levels of genomic modification, hit confirmation, and strong phenotypic effects [124]. The development of the CRISPR/Cas9 technology has undoubtedly transformed the functional genetic analysis in mammals. Recent studies have applied the CRISPR/Cas9 technology to ablate expression of previously identified receptors for viral entry, such as the HIV coreceptors CXCR4 and CCR5, leading to resistance to infection in primary cells [125, 126]. An interesting additional application of CRISPR/Cas9 is the direct editing of viral genes important for viral fitness. This approach has recently been used to target HSV-1, CMV, and Epstein-Barr virus (EBV) essential genes, leading to a significant decrease of viral replication [127]. These studies suggest the potential use of CRISPR/Cas9 as an innovative therapeutic strategy, as aspect that will surely be further explored in the near future.

Another prominent example published recently is the identification of host factors that confer susceptibility to the evolutionary related type III secretion systems, T3SS1 and T3SS2, encoded by *Vibrio parahaemolyticus* [128]. The T3SSs are highly complex nanomachines utilized by gram-negative pathogens to inject a variable repertoire of virulence factors into the cytosol of the eukaryotic cells, enabling pathogen adhesion and internalization of modulation of host processes. Interestingly, using genome-wide CRISPR/Cas9 screens, sulfation and fucosylation of cell surface components were identified as host determinants of T3SS1- and T3SS2-mediated cytotoxicity, respectively. The authors hypothesized that interactions between sulfated cell surface molecules such as host proteoglycans and bacterial adhesins act as facilitators of T3SS1 activity, whereas fucosylated glycans on the surface may serve as receptors for T3SS2 components necessary for insertion of the complex in the host membrane [128]. The CRISPR/Cas9 approach has just started to reveal its power as a tool for unbiased identification of novel ePPIs, elegantly exemplified by the identification of CD300lf as the cell surface receptor for noroviruses, which, strikingly, was identified as the main determinant for the tropism of the murine norovirus [42]. Further optimization of this technology will unequivocally signify a tremendous advance for the discovery of extracellular host-pathogen PPIs, the processes underlying host-pathogen interactions and its possible therapeutic applications.

5.3. Haploid Genetic Screens. Haploid cells, in turn, allow the study of recessive phenotypes that can be masked in diploid cells, due to the difficulties of creating true genetic knockouts in mammalian cells. Despite yeast being a useful tool due to the simplicity of obtaining relevant mutants at its haploid life stage, the majority of human pathogens do not replicate in yeast therefore limiting the applicability of this approach [129]. In recent years, human haploid cells have

been increasingly utilized for genome-wide loss-of-function genetic screens using insertional mutagenesis [43, 44, 47]. In initial studies, Carette and colleagues took advantage of the KBM7 cell line, a derivative of the chronic myeloid leukemia cell line (CML) with a haploid karyotype except for chromosome 8 [130]. Using gene-trap retroviruses for efficient insertional mutagenesis, the authors generated a genome-wide collection of null mutants for most nonessential genes [43]. This approach was successfully utilized to identify host factors essential for the functions of the distending toxins or CDTs, potent virulence factors secreted by a number of pathogenic bacteria. In particular, mutagenized KBM7 cells were treated with *Escherichia coli*-derived CDTs and resistant clones were isolated, leading to identification of insertions in the sphingomyelin synthase 1 and the putative G protein-coupled receptor TMEM181, suggesting that this molecule may serve as a surface receptor for the toxin [43, 44]. Similar haploid screens have identified novel receptors for a number of bacterial toxins, including the lipolysis-stimulated lipoprotein receptor for the *Clostridium difficile* transferase [45], or the low-density lipoprotein receptor-related protein 1 as a host receptor of the *Clostridium perfringens* TpeL toxin [46].

In a later study, Carette et al. generated a KBM7-derived cell line named HAP1, haploid for all chromosomes [47]. Similarly to previous studies, the authors used the retroviral gene-trap approach to mutagenize HAP1 cells followed by deep sequencing to map more than 800.000 insertions. In this study, using a replication competent vesicular stomatitis virus (VSV) carrying the Ebola virus glycoprotein, a previously unknown entry receptor for Ebola virus was identified. Notably, these haploid cell screens identified six members of the HOPS complex, proteins known to play functions in endosomal/lysosomal trafficking, as well as the Niemann-Pick C1 (NPC1) transporter as the most prominent hit of the assay. It is worth noting that NPC1 is not a surface molecule but rather an endosomal receptor. These findings led the authors to propose a novel mechanism of entry by which Ebola virus is internalized into the endocytic pathway, followed by endosome maturation and cleavage of the surface glycoprotein of the virus. Endosome fusion, mediated by the HOPS complex, would allow interaction with NCP1 containing endosomes, triggering fusion and release of the viral genome into the cytosol. Multiple cell surface receptors can lead to internalization of the Ebola virus into the endocytic pathway [131]; such redundancy in receptor usage likely explains why these receptors were not identified in the haploid cell screen [47]. Notably, independent studies have confirmed that NCP1 acts an intracellular receptor for Ebola, including a chemical screen approach, a study showing NCP1 dependence for infection of otherwise nonsusceptible cells, and more recently the elucidation of the crystal structure of this receptor bound to the Ebola virus glycoprotein [132–134].

Interestingly, after the aforementioned haploid genetic screens identified NCP1 as a noncanonical entry receptor (given its intracellular localization), other filoviruses have been shown to take advantage of this receptor [135]. The relevance of this intriguing mechanism of viral entry is further reinforced by recent work on Lassa virus, an Old World Arenavirus that, similarly to Ebola virus, causes severe to fatal hemorrhagic disease in humans [48, 136]. A genome-wide haploid screen using VSV pseudotyped with Lassa glycoprotein was performed in order to identify host factors essential for viral entry. Although α-dystroglycan (DAG1) was long recognized as the cell surface receptor for Lassa virus, additional factors were suspected, given the observation that certain DAG1-expressing cells are resistant to infection. The authors elegantly demonstrated that at a neutral pH, the Lassa virus glycoprotein was bound to DAG1, whereas upon exposure to lower pH (resembling the lysosome environment), a receptor switch occurred leading to strong association with the lysosomal-associated membrane protein 1 (LAMP1) [48]. Thus, similarly to Ebola virus, in the model suggested the virus would be incorporated into the endocytic pathway after interaction with its surface receptor DAG1, followed by increasingly acidic conditions that would result in interaction with LAMP1 in the lysosomal membrane, triggering membrane fusion and release of the virus in the cytosol [48].

More recently, Pillay and colleagues applied the haploid cell screening approach to the identification of host factors essential for the adeno-associated virus (AAV) serotype 2 infection, one of the leading vectors for virus-based genes therapies [49]. Notably, the most significantly enriched gene in these screens was KIAA0319L, a poorly characterized type I immunoglobulin domain-containing transmembrane protein named hereafter as the AAV receptor. Among the 46 host factors identified as hits, many were implicated in heparin sulfate proteoglycan biosynthesis as well as a number of proteins that participate in intracellular transport processes. AAV is known to attach to the cells using heparin sulfate proteoglycans and hijacks endosomal trafficking to travel to the nucleus upon invasion of the cell; thus the authors hypothesized that these additional factors may influence virus tropism [49].

Altogether, these studies elegantly demonstrate the power of genome-wide screens in human haploid cells and the power of this approach to study virus-host interactions. Future studies should further assess the applicability of this method for general detection of interactions that take place at the pathogen-plasma membrane interface. It will also be important to generate additional haploid cell lines, in order to broaden the range of pathogens and pathogen-derived molecules that can be studied using these genetic tools. In this regard, a number of haploid cell lines have been generated in mammals [137], unique tools to elucidate the basic aspects of human genetics.

5.4. Population Genomics for Pathogen-Host Interaction Discovery. Pathogens are among the most intriguing and prominent drivers of human evolution. Humans have adapted to the pressure imposed by microorganisms through genomic diversification, particularly through variation of genes involved in immune system function, constantly challenged by the rapidly coevolving pathogen genomes. The advent of new technologies such as next-generation sequencing and the computational tools associated have opened new avenues for the study of human genetics, making it

possible to evaluate the contribution of genetic diversity to susceptibility to infection at the genomic level. The emergence of datasets of genomic variation in multiple human populations, as well as pathogen genomes, allows detection of signatures of selection, which can be exploited to identify genes with major roles in immunity (for an excellent review see [138]). Remarkably, the cell surface-expressed receptors are among the most polymorphic gene families in mammals, subjected to strong positive selection and rapid evolution, in many instances possibly driven by pathogen molecules that remain unknown [87, 139–141]. Polymorphisms in receptors and immunomodulatory genes contribute to the natural susceptibility of different individuals to infection [142–144], as illustrated by protection against HIV infection in individuals carrying homozygous polymorphisms in the viral coreceptor CCR5 [145]. The identification and further study of genes under positive selection may represent a mainstream approach to dissect novel genes involved in disease and host-pathogen interaction. A notable example is the identification of glycophorin B as the erythrocyte receptor for *P. falciparum* protein EBL-1 through examination of highly polymorphic genes in populations from malaria-endemic regions [50]. Further population genetics studies promise key insights into novel immunological mechanisms and have the potential to provide molecular details that will ultimately help design effective therapies.

5.5. Phagemic and Transposon Library-Based Screens. In addition to these encouraging technologies, the generation of phagemic libraries has also represented an important tool for deciphering PPIs, in this case between particular binding partners and the whole genome of specific pathogens [146–148]. Typically, pathogen-encoded molecules are expressed as fusions with phage envelope proteins, a method known as phage display that has been widely exploited to identify peptides with specific binding properties. For example, Beckmann and colleagues built a phage display library to identify novel group B streptococci proteins capable of mediating adherence to fibronectin, a major component of the extracellular matrix often exploited for colonization of the host [51]. From this analysis, the authors identified 19 genes with homology to known bacterial adhesin proteins, genes involved in virulence, transport, or metabolic processes, along with genes with uncharacterized functions. Interestingly, one of these genes showed significant homology with the ScpB protein, a peptidase found in other streptococci that inactivates the member of the human complement system C5a, suggesting that this bacterial molecule acts as a bifunctional protein, similarly to other examples of multifunctional proteins discussed above [51].

More recently, a transposon-based insertion-inactivation mutant library was elegantly utilized to identify a bacterial protein capable of targeting the surface receptor TIGIT, an inhibitory molecule present in natural killer (NK) cells and T cells [52]. *Fusobacterium nucleatum* is a common oral bacterium that has been associated with colon adenocarcinoma and rheumatoid arthritis among other malignancies. In this study, Gur and colleagues showed that different strains of *F. nucleatum* blocked NK-mediated killing of human tumors. Using a library of *F. nucleatum* mutants, the authors identified Fap2 as the bacterial protein that directly interacted with TIGIT, leading to inhibition of NK cytotoxicity and downregulation tumor-infiltrating T lymphocytes activation. Immunoevasion is a hallmark of cancer; however whether members of the microbiome found within the tumor provide cancer cells with immunoregulatory properties has remained a major matter of debate [149]. These interesting findings suggest that *F. nucleatum* present in the tumor niche may enhance tumor escape by inactivating NK-mediated killing upon interaction of the fusobacterial Fap2 with the inhibitory receptor TIGIT. Of note, transposon-based mutant libraries are readily available for other pathogenic bacteria and have been successfully applied to identification of bacterial genes implicated in bacterial physiology [150–152]. It would be of interest to employ these libraries for unbiased identification of ePPIs. Notably, as mentioned above, we found that HAdV immunomodulators preferentially target other immunoreceptors that, similarly to TIGIT, also play inhibitory functions [28], suggesting this might represent a common immunosuppressive tactic evolved by pathogens. In fact, there is emerging evidence suggesting that may be the case, as a number of extracellular proteins from unrelated human pathogens have already been shown to target diverse immune receptors with inhibitory functions [28, 52, 82, 87, 153, 154]. Further exploration of inhibitory receptor targeting by other pathogens warrants exciting biological discoveries.

6. Concluding Remarks

Deciphering the human genome made possible the categorization of genes that encode for the human secretome; now, the challenge of the postgenomic era is to annotate the functions of those genes and their expression patterns during health and disease. A lot has been learnt from painstaking, highly focused experiments using classical biochemistry. In recent years, the impressive technological advances in proteomics, functional genomics, and computation have revolved our understanding of cell communication and function and have collectively created a versatile platform to enable biological discoveries, from mechanistic explorations to big data and systems biology analysis. Nevertheless our understanding of the molecules and mechanisms of extracellular immunomodulation and pathogen invasion remains remarkably limited.

Extracellular PPIs between host- and pathogen-encoded molecules orchestrate an enormous diversity of cellular processes, from initial colonization of the target cell to subsequent immune responses. The elucidation of these extracellular interactomes is integral to understanding the molecular basis of infection and will guide the development of more efficient or innovative therapeutics. Improvements in proteomics and genomics approaches have exponentially increased our understanding of how pathogens, particularly viruses, modulate the intracellular environment of the cell. Concomitantly, we and several other groups have implemented technologies directed towards elucidation of extracellular interactomes [2, 9, 10, 28, 32, 155], which have begun to reveal fundamental principles of extracellular

host-pathogen interactions. Notably, recent studies have revealed extensive ePPI networks in model organisms such as *Drosophila* or zebrafish [2, 9]. These undertakings predict that, similarly to intracellular PPIs, extracellular networks will be highly connected, with secreted and plasma membrane-expressed proteins having multiple binding partners. However, as discussed in this review, the identification of the host factors and in many cases the pathogen molecules that mediate ePPIs have largely defied molecular identification, in part due to the technical difficulties inherent to the study of these extracellular proteins. The elucidation of the global principles dictating extracellular pathogen-host PPIs will require a coordinated effort to bring together the areas of biology and technology. There are now considerable opportunities for integrating multiple disciplines for ePPI discovery, particularly proteomics and CRISPR/Cas9 genome-wide screens, which should be powered by commensurable advances in bioinformatics and computation for big data analysis. The integration of orthogonal datasets coming from multiple "omics" approaches will be advantageous for elucidating the intricacies of the host-pathogen extracellular interactomes and will further enhance the rational identification of novel therapeutic targets by uncovering fundamental principles of biology.

The journey from classical biochemical studies towards a systems biology approach has just begun and promises major technological breakthroughs and surprising biological findings. The development of powerful technologies for ePPI discovery has already illuminated sophisticated and sometimes unexpected molecular mechanisms by which pathogens interact with their hosts and has provided unique opportunities to increase our understanding of viral and bacterial pathogenesis. Further improvement of these technologies is warranted and will surely provide the scientific community with unprecedented insights into pathogen and host biology.

Abbreviations

AVEXIS: Avidity-based extracellular interaction screen
AAV: Adeno-associated virus
CAR: Coxsackievirus receptor
CMV: Cytomegalovirus
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9
EBV: Epstein-Barr virus
ECD: Extracellular domain
ePPI: Extracellular protein-protein interaction
HAdV: Human adenoviruses
HCV: Hepatitis C virus
HIV: Human immunodeficiency virus
HSV: Herpes simplex virus
ITIM: Immunoreceptor tyrosine-based inhibitory motif
IVTT: In vitro transcription/translation
LAIR1: Leukocyte-associated immunoreceptor like 1
LILRB1: Leukocyte immunoglobulin-like receptor 1
MPA: Microfluidic-based comprehensive human membrane protein array
MPZL1: Myelin protein zero-like protein 1
MS: Mass spectrometry
NAPPA: Nucleic-acid programmable protein array
NCP1: Niemann-Pick C1
PDGFRa: Platelet-derived growth factor receptor alpha
PPI: Protein-protein interaction
PVR: Poliovirus receptor
SARS-CoV: Severe acute respiratory syndrome coronavirus
SV40: Simian virus 40
SLAM: Signaling-lymphocytic activation molecule
SPR: Surface plasmon resonance
T3SS: Type III secretion system
VAVC: Vaccinia virus.

Competing Interests

The author declares that they have no competing interests.

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Review Article

IL-10: A Multifunctional Cytokine in Viral Infections

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The anti-inflammatory master regulator IL-10 is critical to protect the host from tissue damage during acute phases of immune responses. This regulatory mechanism, central to T cell homeostasis, can be hijacked by viruses to evade immunity. IL-10 can be produced by virtually all immune cells, and it can also modulate the function of these cells. Understanding the effects of this multifunctional cytokine is therefore a complex task. In the present review we discuss the factors driving IL-10 production and the cellular sources of the cytokine during antiviral immune responses. We particularly focus on the IL-10 regulatory mechanisms that impact antiviral immune responses and how viruses can use this central regulatory pathway to evade immunity and establish chronic/latent infections.

1. IL-10 and the Complex Interplay between Its Cellular Sources and Targets

Antiviral immune responses ideally eliminate replicating virus and viral reservoirs without host damage. However, in many infections, severe complications could occur due to excessive immune activation. To prevent host tissue damage, immunoregulatory cytokines control the magnitude of these immune responses. IL-10 is a key component of this cytokine system that regulates and suppresses the expression of proinflammatory cytokines during the recovery phases of infections and consequently reduces the damage caused by inflammatory cytokines [1, 2]. IL-10 binds IL-10R, a dimeric receptor composed of a high affinity IL-10R1 chain predominantly expressed on leukocytes and unique to IL-10 recognition, and an ubiquitously expressed IL-10R2 chain involved in the recognition of other cytokines from the IL-10 family (IL-22, IL-26, IL-28A, IL-28B, and IL-29) [3, 4]. The interaction of IL-10 with IL-10R triggers the Jak-STAT signaling pathway, leading to STAT1, STAT3, and, in some instances, STAT5 activation. STAT3 is critical for IL-10 effects on immune cells [5–7].

As its specific receptor (IL-10R1) expression indicates, IL-10's broad spectrum of cellular targets includes virtually all leukocytes. IL-10 is considered a master negative regulator of inflammation. Blockade in the IL-10 pathway typically results in prolonged and exaggerated immune responses to antigens that can lead to immunopathology. Initially identified as a Th1 inhibitory factor secreted by Th2 cells [8], IL-10 is now known to be produced by a variety of innate and adaptive immune cells, including macrophages, dendritic cells (DCs), natural killer (NK) cells, CD4, CD8, $\gamma\delta$ T cells, and B cells (reviewed in [4, 9, 10]). Untangling the complex interplay between IL-10 sources and target cells during immune responses remains an outstanding challenge. For instance, systemic administration of IL-10 for autoimmune therapy proved to be paradoxically proinflammatory [11, 12], whereas localized IL-10 delivery usually proves to be therapeutic [13–15]. Spatial delivery of IL-10 signaling is therefore crucial to its effects.

Autoimmune disease models in IL-10-deficient mice have helped elucidate the role of this cytokine in T cell homeostasis in the periphery. They also highlight the complex link between IL-10's source and its role. IL-10-deficient mice develop spontaneous enterocolitis typically driven by microbial insult and dependent on T cell responses [16–18]. When these mice are bred in pathogen-free environments or when MyD88 (a key component for pathogen recognition receptor (PRR) signaling) is also knocked out, colitis does not occur implicating the gut microflora as a causal agent [16–20]. IL-10 thus maintains T cell tolerance to commensal

microflora in the gut. Treg cells are critical in the prevention of spontaneous colitis in this model [21, 22]. When IL-10 deficiency is restricted to the Treg cell compartment, mice develop colitis [22]. Although Treg cells are the source of IL-10 that maintains peripheral tolerance, they also need to sense IL-10 to provide protection, as IL-10R-deficient Treg cells cannot impair disease development [23]. Restricting IL-10 deficiency to myeloid cells does not cause colitis which confirms that macrophages are not the main source of protective IL-10 in this model [24]. IL-10 produced by macrophages could however partly contribute to colitis protection, as it triggers Treg cell protection when anticommensal T cells are adoptively transferred into a sensitive host [25]. Importantly, deficiency in IL-10R signaling in macrophages leads to colitis development [24, 26]. IL-10 signaling appears necessary for macrophages to trigger their anti-inflammatory functions. Macrophages thus act as intermediates in the maintenance of tolerance. IL-10 produced during the initial inflammation in the gut probably drives IL-10 production by Treg cells, which in turn limits macrophage-induced activation of anticommensal T cells, maintains peripheral T cell tolerance, and controls immunopathology.

This well-studied autoimmune model shows how IL-10 produced locally acts as a natural negative feedback mechanism that controls inflammation and maintains immune homeostasis in the periphery. Indeed, IL-10 deficiency aggravates several experimental autoimmune disorders [27–29], illustrating the central role of this cytokine in immune regulation.

IL-10 is also important in controlling viral immunity. Studies using lymphocytic choriomeningitis virus (LCMV) infections with strains that provoke either acute or persistent infections have helped understand the role of IL-10 in viral infections. IL-10 acts as an immunoregulator, inhibiting proinflammatory responses from innate and adaptive immunity and preventing tissue damage due to exacerbated adaptive immune response. However, viruses have evolved mechanisms that exploit the immunoregulatory function of IL-10 for immune evasion, suppression, and tolerance, promoting their own survival. As a result, viruses can persist for life in infected hosts possessing otherwise competent immune responses. The effects of pleiotropic IL-10 during the course of infection are nonetheless multiple and the subtle IL-10-governed mechanisms that balance inflammation and immunoregulation are still subject to plenty of attention. In this review, we will discuss the role of IL-10 in immune cells during acute infections and the IL-10-dependent mechanisms that viruses use to drive viral persistence.

2. IL-10 in Acute Viral Infection

2.1. Early IL-10 Induction and Effects on Innate Immunity. During the early phase of infections, viruses typically trigger PRR engagement after pathogens-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) recognition (reviewed in [30]). PAMP and DAMP recognition drives the antiviral state in antigen-presenting cells (APC) and type I IFN production that initiate the innate immune response. Concomitant to the proinflammatory first line of defense triggered by PRR signaling, the immunoregulatory cytokine IL-10 is induced in DCs and macrophages (Figure 1) [31–37]. The regulation of IL-10 production in APC is complex and depends on cell type [37] and the integration of secondary activation signals such as type I IFN [34, 38], PGE_2 [39], or CD40 ligation [40] that synergize with PRR signals. Moreover, IL-10 production in APC can be antagonized by the presence of IFN-γ [34, 41]. In macrophages, IL-10 production can be maintained through an autocrine IFN-β feedback loop [36]. In DC, IL-10 production depends on subtype-specific preprogrammed cytokine patterns [37, 40]. Kinetic studies indicate that IL-10 could be produced in late activation phase in APCs [33, 34], which suggests that IL-10 balances the proinflammatory signals induced by viral PAMPs. Early IL-10 production by APCs probably limits excessive inflammation and thus potential tissue damage.

NK and NKT cells are an essential effector arm of innate immunity that participates in the control of viral infections [42–45]. IL-10 has been shown to promote NK cell proliferation, cytokine production, and cytotoxicity in vitro [46–50], although in some in vivo settings it could modulate NK cell activity [51, 52]. IL-10 acts as a prosurvival factor in activated NK cells by inhibiting activation-induced cell death [53]. The cytokine thus appears to promote activated NK cell effector function. Interestingly, NK cells are also a source of IL-10 upon synergistic activation with IL-2 and IL-12 (Figure 1) [54–57]. IL-10-producing NK cells can control liver inflammation in acute murine cytomegalovirus (MCMV) infection [58] and therefore limit immunopathology in some organs. IL-10-producing NK cells could serve as an early control for excessive inflammation during the initiation of the immune response [59, 60], while their viremia-controlling effector functions are maintained. IL-10 produced in the early phase of antiviral innate immunity by APCs and NK cells is probably a counterbalance to proinflammatory signals that protect from tissue damage. Although in most cases IL-10 derived from innate immune cells is unlikely to affect the development of antiviral immunity, this source of IL-10 can be induced by some viruses to evade immunity, as described later.

2.2. IL-10 and Antiviral Cellular Responses. To eliminate intracellular pathogens like viruses the immune system typically uses cytotoxic $CD8^+$ T lymphocytes (CTL), whose functions are armed by Th1 cells. $CD8^+$ T cells are critical in antiviral immunity, since they can kill infected cells through the recognition of viral peptides presented on MHC I molecules. Th1 cells also recognize viral peptides presented by APC on MHC-II molecules. Th1 cells provide the "license to kill" to the virus-specific $CD8^+$ T cells to differentiate into effector CTLs using professional APC as intermediates [61, 62]. This central mechanism of antiviral immunity can be modulated by IL-10 at different levels. High IL-10 levels act as a regulatory trigger that initiate the resolution of the acute phase of infection in which antiviral T cell populations contract [63].

2.2.1. IL-10 Production by Antiviral T Cells. Currently it is well established that virtually all T cell subsets can produce

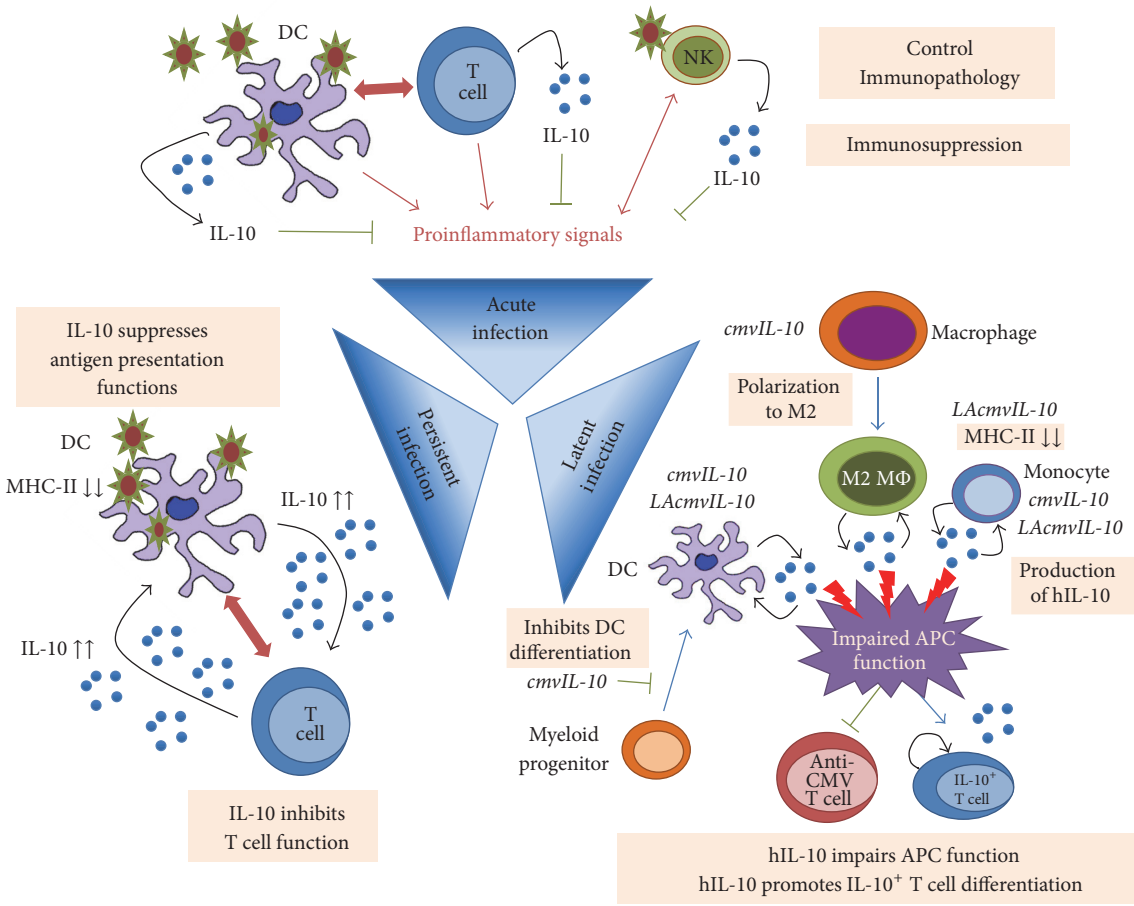

Figure 1: IL-10 role in viral infections. During acute infections, proinflammatory signals are produced by DCs after recognition of pathogen patterns. In parallel, NK cells recognizing pathogen patterns and/or stimulated by proinflammatory signals further enhance inflammation. In this proinflammatory context, DC can promote antiviral T cell responses that clear the infection. Activation of DC, T cells, and NK cells also results in the production of the immunoregulatory cytokine IL-10 to balance inflammation. In this context, IL-10 expression controls immunopathology and leads to the resolution of the inflammation and T cell responses once the pathogen is cleared. During persistent infections, the virus exploits the production of IL-10 by DCs to exhaust antiviral T cells. High IL-10 levels produced by DCs suppress their antigen presenting capacity and lead to inefficient T cell activation. Chronic antigen presence further exhausts T cells and induces IL-10 production. T cells therefore become "tolerant" to viral antigens and infection persists. To establish chronicity and latent infections, the virus produces viral IL-10 homologs that favor anti-inflammatory responses. In human cytomegalovirus infection, cytomegalovirus-encoded IL-10 (cmvIL-10) and latency-associated cytomegalovirus-encoded IL-10 (LAcmvIL-10) are produced in myeloid cells and impair their function. cmvIL-10 induces hIL-10 production in DCs, macrophages, and monocytes, impairs DC differentiation, and promotes M2 polarization of macrophages. LAcmvIL-10 also promotes hIL-10 production in DCs and monocytes and impairs monocyte presenting capacity. IL-10 viral homologs induce human IL-10 (hIL-10) production in myeloid cells that contributes to impairment of their antigen presenting cell (APC) function. This in turn probably limits anti-CMV T cells responses and promotes IL-10^+ T cell development. Impaired APC function permits chronic infections, while IL-10^+ T cells allow latent infections to persist.

IL-10 (reviewed in [64, 65]). IL-10 production appears thus to be embedded in the activation program of T cells. Indeed, at the height of the inflammatory response and once cellular immune responses are mounted, antiviral $CD4^+$ and $CD8^+$ T cells become the main sources of IL-10 (Figure 1) [66–72]. Th1 cells can produce IL-10 [73] in response to intracellular protozoan [74, 75], LCMV [72, 76], MCMV [77–79], or influenza [68] infections among others. IL-10 production in Th1 cells is driven by TCR engagement but is not directly regulated by T-bet, the master transcription regulator of Th1 cell programming [80, 81]. IL-27 (a proinflammatory cytokine belonging to the IL-12 family) is a potent inducer of IL-10 in Th cells [82–85]. Type I IFN can also induce IL-10 expression in $CD4^+$ T cells [86, 87]. IL-10 production in Th cells therefore depends on secondary environmental signals upstream of STATs (such as IL-10 itself [5–7] and proinflammatory cytokines [88]) or SMADs (such as TGF-β [89]). It should be noted that chronic antigen stimulation results in IL-10-producing Th1 cells [72, 90, 91] unable to respond to pathogens. This natural regulatory mechanism that maintains T cell homeostasis in the periphery can be used to establish chronic infection as discussed later.

Effector $CD8^+$ T cells can produce IL-10 during the acute phase of influenza virus [67, 68], respiratory syncytial

virus [70], coronavirus infection [69], paramyxovirus simian virus 5 [71], or vaccinia [66] infections. The transcription factor BLIMP-1 is essential for IL-10 production in effector and memory $CD8^+$ T cells [92]. BLIMP-1 is induced in $CD8^+$ T cells through T cell help and can be sustained by proinflammatory signals (IL-27), T cell growth factors (IL-2) [92], and antiviral signaling like type I IFN [67]. It thus appears that antiviral and inflammatory signals elicited during viral infections trigger activated T cells to produce IL-10 as a feedback regulatory mechanism that limits excessive inflammation.

2.2.2. IL-10 Uses APC as Intermediate to Modulate T Cell Responses. Although T cells become the main IL-10 produ cers during the acute phase of infection, IL-10 effects on T cell function are usually mediated through paracrine activity on DCs and macrophages (reviewed in [1, 9]). IL-10 recognition by APC skews their response towards a noninflammatory protissue repair phenotype [93–99]. IL-10 is a major regulator of the potent APC-derived inflammatory cytokine IL-12 [100] and promotes expression of its own mRNA in a positive feedback loop [101]. Exposure to IL-10 also leads to down-regulation of costimulatory and MHC molecules on APCs [4, 102, 103] which limits the amount of antigen exposure T cells can receive. IL-10 also restricts the production of proinflammatory cytokines and chemokines that permit APC trafficking to the lymph nodes, thereby interrupting Th1 differentiation of naïve T cells [103, 104]. These elevated IL-10 levels impair de novo Th1 stimulation [105, 106] and trigger the resolution of the acute phase of infection in which antiviral T cell populations contract [63]. IL-10 therefore acts as a switch on APC that controls inflammation and ultimately interrupts T cell responses once pathogens are cleared.

2.2.3. IL-10 Effects on Antiviral T Cells. Through its effects on APC, IL-10 can alter antiviral T cell function, although its effects on Th1 cells and CTLs are very different. Acute and chronic LCMV infection models have been essential to comprehend IL-10's crucial role in controlling antiviral T cell responses. IL-10 limits cytokine production and proliferation in antiviral Th1 cells [2, 104, 107]. When IL-10 regulatory action is removed (through IL-10R blockade or IL-10 deficiency), antiviral Th1 responses can prevent chronic LCMV infection [2, 31, 104, 107, 108]. IL-10 blockade increases the amount of Th1 cells in germinal centers [104], promotes Th1 priming [106], and enhances Th1 effector function and memory development [104, 107]. IL-10 thus appears central to the regulation of antiviral Th1 cell responses. Removal of the IL-10 "brake" on Th responses can lead to immunopathology following viral infection as illustrated by the increased neurologic disease detected in IL-10-deficient mice during fatal alphavirus encephalomyelitis [109]. This general regulatory mechanism prevents host immunopathology and controls the amplitude of Th1 cell responses during acute viral infections. This mechanism can nonetheless be exploited by viruses to promote chronic and persistent infections as discussed later.

In contrast to Th1 cells, $CD8^+$ T cell effector functions (e.g., cytokine production and cytotoxicity) can be enhanced by IL-10 addition in vitro [4]. IL-10 blockade prior to LCMV infection only results in a modest increase in LCMV-specific $CD8^+$ T cells 8 days after infection [104, 107], which indicates that IL-10 does not greatly alter antiviral $CD8^+$ T cell priming. Nonetheless, IL-10 blockade/deficiency facilitates virus clearance by $CD8^+$ T cells in chronic LCMV infections [2, 31, 104, 107], which confirms that secondary $CD8^+$ T cell responses are regulated by IL-10 [105]. It should be noted that the effects of IL-10 on $CD8^+$ T cells could also depend on the strength of the antigenic signal, as $CD8^+$ T cells recognizing different LCMV epitopes appear to have different IL-10 inhibition thresholds [104].

IL-10 has also been linked to $CD8^+$ T cell memory differentiation [110, 111]. Recently IL-10 produced by Treg cells was shown to promote $CD8^+$ T cell memory differentiation in LCMV infections by insulating a portion of $CD8^+$ T cells from inflammatory signals during the resolution phase of the immune response [112]. Other reports have nonetheless indicated that IL-10 could impair $CD8^+$ T cell memory development in the same infection [104], while others found no difference in the quality and quantity of $CD8^+$ T cell memory development after IL-10 blockade [107]. These contradictory results obtained through different approaches (IL-10/IL-10R antibody blockade, IL-10-deficient mice, or adoptive transfer of IL-10-sufficient Treg cells) hint at a very delicately regulated system for CD8 memory development that could be controlled by T cell signal strength as well as spatial and temporal IL-10 delivery. This raises the intriguing possibility for a new facet in IL-10 biology whereby IL-10 dampening of $CD8^+$ T cell responses could facilitate the differentiation of a portion of these cells into memory.

2.3. IL-10 and Antiviral Humoral Response. B cell-produced antibodies represent the other major arm of the adaptive immunity involved in virus clearance [113]. Most clinically effective vaccines not only require the induction of cellular immunity but also the production of neutralizing antibodies [114, 115]. Nonneutralizing antibodies can also participate in antiviral immunity as shown in LCMV infections where virus-specific nonneutralizing antibodies participate in virus clearance alongside $CD4^+$ and $CD8^+$ T cells [108]. The importance of B cell responses in viral immunity is also exemplified by the interference of viruses with humoral immunity. For instance, Bluetongue virus can affect antiviral antibody titers early in infection [116], and human immunodeficiency virus (HIV) can continuously mutate its antigenic determinants, a phenomenon known as antigenic drift, to evade neutralization by antibodies [117, 118].

Since IL-10 regulates B cell survival and differentiation [4], it could potentially control B cell responses to virus. IL-10 favors B cell effector function by stimulating plasma cell differentiation at the expense of B memory cells [4, 119]. Autocrine IL-10 production promotes B cell survival and Ig class switch [120–122]. In LCMV-infected mice, IL-10 however does not control B cell differentiation in the priming phase [104]. Moreover IL-10 blockade does not affect follicular Th cell numbers, a subpopulation of Th cells involved in B cell help and necessary for the generation of high affinity antibodies [104]. It thus appears that IL-10 may

not directly affect B cell responses, although this has not been widely studied.

B cells could nonetheless be a source of IL-10 that could modify antiviral responses. IL-10 expression in B cells can be triggered by TLR engagement [123–125] and increases when B cells are activated in a context mimicking T cell and DC help, that is, through anti-Ig antibody, anti-CD40 antibody, and IL-12 [126]. Type I IFN that are typically produced during antiviral responses can also enhance TLR-induced IL-10 production in B cells [127, 128]. These reports indicate that IL-10 production is an integral part of B cell activation programming. However, the factors driving IL-10 production in B cells during immune responses are not fully understood.

A B regulatory cell population (Breg) has been described [129, 130] and can be a principal source of IL-10. No precise Breg cell markers have so far been defined (reviewed in [131, 132]), but these cells are potent inhibitors of autoimmune inflammation through their IL-10 production [133, 134]. Breg cells can suppress *Listeria monocytogenes* [135] or *Salmonella typhimurium* [136] clearance. These cells can therefore also modulate responses to infections. The observation that Breg cells can be therapeutic in allergy [137] indicates that IL-10 produced by Breg cells could have systemic activity. IL-10-producing Breg cell numbers increase in coxsackie virus-induced acute myocarditis model [138]. In MCMV murine infections, IL-10 expression in B cells can suppress MCMV-specific $CD8^+$ T cells responses [139, 140]. Moreover, IL-10-producing Breg cells could promote chronic MCMV brain infection [141]. In HIV patients, IL-10-producing Breg cells are elevated in peripheral blood of untreated patients and can suppress virus-specific $CD4^+$ and $CD8^+$ T cell activity in vitro [142]. Similarly, in chronic hepatitis B virus (HBV) patients, IL-10-producing B cells are elevated in the periphery and suppress HBV-specific $CD8^+$ T cell responses [143]. IL-10-producing B cells have therefore the capacity to create an immunoregulatory milieu unsuitable for cellular immunity. The localized effects of IL-10 on T cells however suggest that B cell-derived IL-10 would probably affect effector T cell activity in specific settings. Further work will be required to clarify both how B cell-derived IL-10 influences antiviral responses and how IL-10 modulates antiviral B cell responses.

2.4. IL-10 and Virus Clearance. Although IL-10 acts as an immune brake on inflammation, its overall effects on antiviral immune responses can be complex and depend on the virus, site of infection, timing of the antiviral immune response, and so forth. For instance, high IL-10 plasma levels could be protective in early responses to HIV but become detrimental during acute infection as they promote virus persistence [144].

In some settings, IL-10 expression can contribute to virus clearance. In influenza infections, coproduction of IL-10 and IFN-γ facilitates anti-influenza antibody accumulation in the lung mucosa [145]. Thus IL-10 not only limits immunopathology in this case but also supports adaptive immunity. In cutaneous vaccinia virus infections, IL-10-producing T cells have been linked to lesion control [66], which suggests that local IL-10 effects may be multiple and depend on the organ and microenvironment.

IL-10's supportive role for effective virus clearance is very apparent in CNS infections. Virus-induced encephalitis results from an excessive immune-induced inflammation designed to control viral infection. IL-10-deficiency aggravates this immunopathology in *Flavivirus* or *Coronavirus* infections with CNS tropism [63, 146–149]. In these CNS infections, IL-10 usually improves virus control, although this outcome probably results from direct and indirect effects of the cytokine. In CNS immune responses to the coronavirus mouse hepatitis virus, $CD4^+$ T cells and $CD8^+$ T cells are the initial sources of IL-10 [63]. Once the viral load is controlled, IL-10-producing $CD8^+$ T cells diminish while IL-10-producing $CD4^+$ T cells remain [63]. IL-10 produced during the immune response peak could enhance CD8 activity while limiting APC-driven inflammation. During this resolution phase, natural $CD4^+$ $CD25^+$ Treg cells are the main source of IL-10. However transition in the IL-10 source from natural Treg cells to T regulatory 1- (Tr1-) like $CD4^+$ $CD25^-$ cells could be a sign of CNS viral persistence [63] and indicate chronic antigen stimulation. In infection with the *Flavivirus* Japanese Encephalitis virus, IL-10-producing $CD4^+$ $Foxp3^+$ natural Treg cells improve survival in a murine model probably by controlling the immunopathology [148]. In other organs, modulation of immunopathology by IL-10 during infection is not solely reliant on Treg cell activity. In MCMV acute infection, NK cells are the main IL-10 source that modulates immunopathology in liver [58], while IL-10-producing Breg cells probably participate in neuroinflammation control [141]. IL-10 regulatory mechanisms are therefore essential to control severe inflammatory responses produced by viral infections and can thereby, albeit indirectly, be essential for virus clearance.

In an adequate acute immune response, IL-10 presence should not affect virus clearance; however sustained expression during immune priming or secondary responses can favor persistence or chronic infections. This fine balance between the inflammatory response crucial to virus clearance and the IL-10-mediated immune regulation necessary for T cell homeostasis and host tissue protection can be subverted by viruses to allow replication and spreading.

3. IL-10 in Chronic Viral Infections

Persistent or chronic viral infections are not cleared by the host immune response and result in long-term equilibrium between the host and the virus. Several factors can contribute to this persistence such as viral immune evasion mechanisms, impaired viral clearance facilitated by the host-regulated immunosuppression, or, as for herpesviruses, manipulation of the host immune environment to enable persistence (latency). We will next review different mechanisms used by viruses to induce chronicity or persistence, in which either host IL-10 is involved as a regulating cytokine or viruses have evolved mechanisms that mimic IL-10 function, such as IL-10 viral homologs.

3.1. Persistent Viral Infections. Persistent infections such as those established by hepatitis C virus (HCV), HBV, and HIV are of particular interest in human health due to their

high rates of morbidity and mortality as well as the lack of efficient therapies. Impaired viral clearance can result from viral evasion of the immune response or be assisted by the host-regulated immunosuppression. More precisely, $CD4^+$ T cells and $CD8^+$ T cells lose their effector functions and are unable to control viral infections, a phenomenon called T cell exhaustion [150] (Figure 1). $CD8^+$ T cells lose the ability to produce antiviral cytokines, to kill infected cells, and to proliferate in response to antigen stimulation [151]. Similarly, $CD4^+$ T cells show impaired cytokine production and lack of proliferation [90]. This loss of T cell function has been described in persistent infections with HCV, HBV, HIV, and LCMV, suggesting that a conserved mechanism of immunosuppression may downregulate T cell function. These mechanisms produce gene expression changes in T cells, including inhibitory receptor induction [152, 153], production of soluble factors such as TGF-β [154], or elevated systemic IL-10 levels [2, 155, 156]. The programmed death-1 (PD-1)/PD-ligand(L)1 inhibitory pathway actively suppresses T cell responses and can also participate in the establishment of persistent infections [106, 152]. Although PD-1 contributes to T cell exhaustion, a common characteristic of these persistent infections is elevated IL-10. This has been described for HCV and HIV infections in which high IL-10 levels in the early/acute phase are associated with progression to persistence [157–160], which suggests that this is an evolutionarily conserved mechanism in persistent viral infections with clinical relevance.

Studies on LCMV persistent infection have helped elucidate the mechanisms by which IL-10 can mediate persistent infections. Infection of adult mice with Armstrong (Arm) LCMV strain results in acute infections that are efficiently cleared within 7–10 days by anti-LCMV $CD8^+$ CTLs. By contrast, the LCMV clone 13 (Cl13) induces a persistent infection that suppresses cellular and humoral responses. Cl13 infection of DCs results in cell loss in this compartment during the first week of infection and plays a relevant role in establishing persistence [161–163]. Among the different host factors that play a role in immunosuppression in Cl13 infections, it has been documented that IL-10 production is highly increased in serum. Neutralization of IL-10 activity by treatment with anti-IL-10R antibody rescues T cell responses and consequently virus clearance occurs [2, 31]. Similarly, Cl13-infected IL-$10^{-/-}$ mice show increased T cell function and viral clearance [2, 31]. Thus, IL-10 induces immunosuppression that leads to viral persistence.

IL-10 mechanism of action in viral persistence involves complex cellular cross-talks and interplay between the cytokine source and its target. IL-10^+ DCs increase in frequency during the acute phase of Cl13 infection and then decline with time [164]. Thus during the acute phase and up to the time that T cell exhaustion is initiated, DCs are the main cellular source of IL-10. Increased IL-10 production by DCs has also been reported during HIV, HCV, and foot-and-mouth disease virus infections, specifically inducing loss of T cell responses [165–170]. Within the DC populations, IL-10 production is higher in $CD8\alpha^-$ DCs and those expressing high CCR7 levels, a receptor required for DC migration to T cell areas in secondary lymphoid tissues [171]. IL-10 production in these DCs therefore increases the likelihood for IL-10 exerting its regulatory influence on T cells. A similar scenario has been described for HIV in which IL-10-induced immune dysfunction has been related to the modification of DC populations able to gain access to areas where the quality of adaptive immune responses can be profoundly modulated [167]. This mistimed virus-induced IL-10 production by DCs therefore promotes persistent/chronic infections by affecting the inflammatory balance necessary to mount effective T cell responses.

In later stages of chronic Cl13 infection in mice (i.e., from day 8 after infection and throughout the course of disease), NK cells and virus-specific T cells also play a large role in producing IL-10 [72]. In the T cell compartment, virus-specific $CD4^+$ T cells become the main IL-10 overproducers. These data are in line with data from other nonviral infections such as *Leishmania* [172], malaria [74], or *Toxoplasma* [75], in which IL-10 produced by T cells has a high impact on disease outcome.

This induction of IL-10 production in $CD4^+$ T cells is probably a homeostatic mechanism that limits Th-induced inflammation [173]. IL-10 is induced in Th1 cells obtained from LCMV-nonchronically infected mice after antigen reexposure [72], and chronic antigen exposure can lead to the differentiation of IL-10-producing self-regulatory Th1 cells [90, 91, 174]. Repeated antigen exposure could thus convert virus-specific Th cells into IL-10-producing self-regulatory Th1 cells, a mechanism that could further feed LCMV chronic infections. These self-regulatory Th1 cells can prevent DC maturation and suppress Th1 cell differentiation [102]. This negative feedback mechanism can thus be used by LCMV to suppress Th1 effector function. Similar to self-regulatory Th1 cells, Tr1-like cells have been identified as the main IL-10 producers in HIV infections [175]. Tr1-like cells can also be generated through repeated TCR stimulation in the presence of IL-10 [176], but only when APC are present in the culture [177]. The DC-T cell cross-talk in the presence of high IL-10 levels can thus give rise to IL-10-producing T cells that limit T cell immunity. Hepatitis C virus (HCV) chronically infected patients show an increase in IL-10 production by NK cells [158]. In this case, IL-10-producing NK cells could produce a DC-NK cell cross-talk that impairs adaptive immune response and contributes to chronic infections. It is thus apparent that chronic viral infections often use the regulatory role of IL-10 on T cells and APC to cause T cell exhaustion and deactivate antiviral T cell immunity. Blockade of IL-10R with antibody treatment rescues T cell function and contributes to clearance of persistent infections, suggesting that therapeutic strategies that neutralize IL-10 activity could help control persistent infections, such as HCV, together with other molecular therapies.

3.2. Viral IL-10 Homologs in Chronic and Latent Infections. Latency is a mode of persistent or chronic infection in which the viral genome is retained in the host cell, but with a profound restriction on gene expression that results in the production of few viral antigens and no viral particles (reviewed in [178]). Under appropriate conditions, the expression of the

viral genome can be induced and infectious particles are produced. To establish latency, viruses have developed immune evasion mechanisms that allow for persistence. Among these mechanisms, large DNA viruses encode for protein homologs of cytokines and chemokines or express viral factors that alter host cytokine production [179, 180]. Members of the representative latency-inducing Herpesviridae family, such as human cytomegalovirus (HCMV) [181], Epstein-Barr virus (EBV) [182], ovine herpesvirus 2 [183], and equine herpesvirus 2 [184], encode for IL-10 homologs. Among the best-characterized IL-10 homologs are the cytomegalovirus-encoded IL-10, termed cmvIL-10, and the latency-associated cmvIL-10, termed LAcmvIL-10 [181, 185] (Figure 1). HCMV is a β-herpesvirus that infects a majority of the world's population. Following primary infection, HCMV establishes a lifelong latent infection in cells of the myeloid lineage from where it can later be reactivated to produce infectious progeny [186]. HCMV success in infecting host's cells and causing disease relies partially on a number of virally encoded proteins that are homologs of cellular cytokines, chemokines, and their receptors [181], in which the IL-10 homolog plays an important role. During productive infection cmvIL-10 transcripts are expressed from the gene UL111A [185, 187]. This gene also encodes for the splice variant LAcmvIL-10, which has been associated with latency [188]. cmvIL-10 protein shares 27% amino acid identity with hIL-10 but retains the ability to bind the hIL-10 receptor [187]. Therefore, cmvIL-10 mediates immunomodulatory functions similar to hIL-10 such as inhibiting proinflammatory cytokine production, decreasing MHC-I and MHC-II expression in monocytes [189], and impairing monocyte-derived DCs maturation [190].

Another immunomodulatory mechanism of action for cmvIL-10 resides in its ability to alter macrophage polarization. Depending on the signal they received, monocytes and macrophages become polarized to either M1 proinflammatory or M2 anti-inflammatory subsets [191]. M1 macrophages have a proinflammatory effect with a relevant role in defense against intracellular pathogens. By contrast, M2 macrophages show increased phagocytic activity and suppress proinflammatory cytokine production. cmvIL-10 modulates macrophage polarization and promotes an M2 phenotype [192] characterized by downregulation of MHC-II, upregulation of molecules associated with anti-inflammatory functions, and poor activation of $CD4^+$ T cells.

Viral IL-10 homologs probably shape the immune response in the early phase of infection by promoting anti-inflammatory signals. cmvIL-10 induces the upregulation of hIL-10 in monocytes, macrophages, and DCs, thereby amplifying IL-10-mediated immunosuppression and favoring chronicity [193]. Viral Rhesus CMV IL-10 homolog is critical for establishing chronic infections, yet during latent phase a better correlation was observed with cell-derived IL-10 levels than with viral homolog [194]. IL-10-producing $CD4^+$ T cells are also linked to HCMV and MCMV persistence [77, 79, 195]. These data indicate that CMV mostly uses endogenous IL-10 signaling to maintain persistence. Taken together these mechanisms enhance the ability of HCMV to establish a primary productive infection and contribute to productive chronic infection.

By contrast, the function of LAcmvIL-10 is much more limited. While both cmvIL-10 and LAcmvIL-10 suppress MHC-II expression on monocytes, LAcmvIL-10 does not impair DC maturation nor does it suppress proinflammatory cytokine production [196, 197]. LAcmvIL-10 can also upregulate hIL-10 in latently infected myeloid cells, although it probably uses a different activation mechanism to cmvIL-10, as LAcmvIL-10 and cmvIL-10 interact differently with the IL-10 receptor and trigger distinct signaling events [196].

Another well-known example of herpesvirus encoding an IL-10 homolog is EBV. EBV is a γ-herpesvirus carried by a high percentage of the human population. EBV infections are mostly asymptomatic, but in some cases EBV induces mononucleosis or B cell and epithelial-cell malignancies [198]. One of the strategies used by EBV to establish latent infections is to produce a viral IL-10 (vIL-10) encoded by the BCRF1 gene, classified as a late gene but expressed in B cells early after infection [199]. vIL-10 has been shown to bind to and signal through the human IL-10 receptor, similarly to cmvIL-10 [200], although its affinity for the IL-10 receptor is 1000-fold lower than that of hIL-10 [201]. The lower receptor affinity of vIL-10 compared to hIL-10 does not allow vIL-10 to stimulate the proliferation of thymocytes or mast cells [202, 203], but it retains the capacity to suppress proinflammatory cytokine production and enhance B-cell viability. During EBV infection vIL-10 seems to play a role only during acute infection, during which it protects infected B cells by altering cytokine production, inhibiting CD4 and NK cell responses, and ultimately facilitating EBV dissemination [199, 204].

4. Concluding Remarks

IL-10's main function is to prevent immunopathology during inflammatory responses. IL-10 can be produced by virtually all immune cells and in turn IL-10 can modulate the response of these cells. Untangling the complex interactions of this pleiotropic cytokine remains an outstanding challenge for immunologists. IL-10 is so central to immune response regulation that viruses exploit this pathway to evade immunity and establish persistent/latent infections. IL-10 effects in the course of viral infections depend on its spatial and temporal delivery. IL-10 can impair T cell priming in the early stages of adaptive immunity, a mechanism that viruses use to promote their persistence by infecting APC and inducing IL-10 production. The effects of IL-10 on the immune response during acute infections are more subtle. The cytokine is produced in high amounts by antiviral effector T cells at this stage. IL-10 prevents tissue damage in this phase while probably not affecting effector function of antiviral $CD8^+$ T cells. IL-10 does however negatively regulate Th1 responses by downmodulating antigen presenting capacity of APC. This regulatory mechanism promotes inflammation resolution when the pathogen clears. Mistiming of IL-10 production at this stage can impair antiviral T cell responses, favoring an early resolution phase that can lead to chronic infection. Chronic antigen exposure in this phase can exhaust antiviral

T cells and switch their phenotype to IL-10-producing cells unable to reactivate when presented again with the antigen.

IL-10 blockade, an attractive therapy to treat chronic infection, should be approached with caution since, for instance, IL-10 can be necessary for virus clearance in CNS infection where it controls immunopathology. IL-10 could also play a role in antiviral $CD8^+$ T cell memory development; thus IL-10 blockade could prove detrimental to establish long-term $CD8^+$ T cell memory. Targeting the fine balance between inflammation and resolution controlled by IL-10 will therefore require spatial and temporal refinement of delivery approaches. A better understanding at the basic level of IL-10 sources and IL-10 effects on the different components of immunity during infections will allow for precise therapeutic targeting of this pathway.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

RNA-Seq Based Transcriptome Analysis of the Type I Interferon Host Response upon Vaccinia Virus Infection of Mouse Cells

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Vaccinia virus (VACV) encodes the soluble type I interferon (IFN) binding protein B18 that is secreted from infected cells and also attaches to the cell surface, as an immunomodulatory strategy to inhibit the host IFN response. By using next generation sequencing technologies, we performed a detailed RNA-seq study to dissect at the transcriptional level the modulation of the IFN based host response by VACV and B18. Transcriptome profiling of L929 cells after incubation with purified recombinant B18 protein showed that attachment of B18 to the cell surface does not trigger cell signalling leading to transcriptional activation. Consistent with its ability to bind type I IFN, B18 completely inhibited the IFN-mediated modulation of host gene expression. Addition of UV-inactivated virus particles to cell cultures altered the expression of a set of 53 cellular genes, including genes involved in innate immunity. Differential gene expression analyses of cells infected with replication competent VACV identified the activation of a broad range of host genes involved in multiple cellular pathways. Interestingly, we did not detect an IFN-mediated response among the transcriptional changes induced by VACV, even after the addition of IFN to cells infected with a mutant VACV lacking B18. This is consistent with additional viral mechanisms acting at different levels to block IFN responses during VACV infection.

1. Introduction

Type I interferons (IFNs) constitute a family of related cytokines (IFN-α subtypes, IFN-β, and other IFN family members) that bind a common and heterodimeric cell surface receptor (IFNAR) and play an important role in the first line of defence against virus infections [1–3]. After initial molecular recognition of the invading virus by host cell pattern recognition receptors (PRRs), these IFNs are secreted and bind cognate cellular receptors to exert their function either locally or distally. This binding initiates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signalling cascade to trigger the activation of diverse host genes, depending on cell type, with potent antiviral activity that contributes to the establishment of an antiviral state in the adjacent healthy cells and the activation of the apoptotic program to eliminate infected cells. Thus, the main purpose of the IFN response is to limit virus replication and infection spreading [4].

Vaccinia virus (VACV) is the most studied member of the Poxviridae family of large DNA viruses with cytoplasmic replication. VACV is the vaccine used to eradicate smallpox more than 30 years ago and constitutes an excellent model to analyze the evasion of the IFN based host response to viral infection. Viruses have to neutralize the antiviral activity of IFNs, and in this sense VACV and other poxviruses seem to be unique encoding a plethora of genes to this effect (reviewed in [2, 3, 5, 6]). Among others, VACV encodes the A46 and A52 protein to inhibit toll-like receptor (TLR) signalling that leads to IFN production [7] and VH1 to dephosphorylate STAT1 and STAT2 [8, 9] but also diverse proteins to specifically inhibit the antiviral activity of some IFN-induced genes. This is the case of the E3 and K3

proteins that employ two different mechanisms to counteract double-stranded RNA-dependent protein kinase (PKR) effector functions [10, 11]. Additionally, E3 binds the product of the IFN-stimulated gene 15 (ISG15) to prevent its antiviral action [12]. But one the most efficient strategies employed by poxviruses to avoid IFN effects is to encode IFN binding proteins that are secreted from infected cells to prevent the interaction of IFNs with their cellular receptors. In the case of VACV strain Western Reserve (WR), the type I IFN binding protein is encoded by the *B18R* gene (*B19R* in the Copenhagen strain). A relevant role of this protein in VACV pathogenesis was soon assigned, since the lack of *B18R* expression after intranasal infection of mice resulted in an attenuated virus, indicating that blocking the IFN host response is crucial for the development of VACV infection [13]. The B18 protein has no amino acid sequence similarity to cellular IFN receptors and, in contrast to the cellular counterparts, binds IFNα/β from a broad range of host species [13]. The protein is synthesized early after VACV infection, is secreted into the medium, and is found as a soluble form or anchored to the cell surface [14, 15]. This binding to the cell surface has been shown to occur *via* interaction of the B18 amino terminus with glycosaminoglycans (GAGs) [16] and allows B18 to prevent the establishment of an IFN-induced antiviral state in cells surrounding the infection site.

In the present study, by using RNA sequencing with the Illumina technology (RNA-seq) and differential gene expression analyses, we have further analyzed the ability of B18 to block the IFN based response in a mouse fibroblast cell line. We also extend the study to VACV-infected cells to identify changes in host gene expression profile induced by VACV or a VACV mutant lacking the *B18R* gene (VACVΔB18), with special emphasis on the inhibition of the type I IFN-induced host cell response.

2. Materials and Methods

2.1. Cell Culture and Reagents. Mouse L929 cells were used to obtain RNA samples for high-throughput sequencing, while BSC-1 cells (African green monkey kidney origin) were used to prepare virus stocks. Recombinant His-tagged VACV B18 protein was expressed in the baculovirus system and purified as previously described [17]. Protein purity was checked on Coomassie blue-stained SDS-PAGE and quantified by gel densitometry. Murine recombinant IFN-α subtype A was purchased from PBL Assay Science (>95% pure), diluted in phosphate-buffered saline, and maintained at −70°C until use.

2.2. Viruses and Infections. Virulent VACV strain WR and the correspondent VACV mutant lacking B18R expression (VACVΔB18, [14]) were grown in BSC-1 cells and stocks of semipurified virus were prepared by sedimentation through a 36% sucrose cushion. L929 cells were infected with VACV or VACVΔB18 with a multiplicity of infection of 5 plaque forming units (pfu)/cell in order to ensure the infection of all cells to obtain a representative RNA-seq profile of each condition. After adsorption of virus for 1 h at 37°C, the virus-containing medium was removed, and cells were washed twice with phosphate-buffered saline and replaced with fresh culture medium supplemented with 2% fetal bovine serum. Infected cells were then incubated at 37°C and harvested at 4 or 8 h postinfection (hpi) by scrapping. Where indicated, IFN (50 units/ml) was added to the infected cultures at 4 hpi and the incubation extended at 37°C to 9 hpi. Inactivation of viruses was performed as previously described [18], by incubation with 2 μg/ml psoralen (4-9-aminomethyltrioxsalen; Sigma) for 10 min and then UV-irradiated for 10 min with 2.25 J/cm^2 in a Stratalinker 1800. Complete inactivation (>10^8-fold reduction in pfu) was confirmed by plaque assay in BSC-1 cells.

2.3. RNA Extraction and Illumina RNA-Seq Library Preparation. Immediately after harvesting the samples, total cellular RNA was isolated from 1.2×10^6 L929 cells using SV Total RNA Isolation System (Promega). RNA samples were quantified on a spectrophotometer (NanoDrop ND-1000; Thermo Scientific) and quality-analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). All samples exhibited a RNA integrity number (RIN) over 9. The sequencing libraries were generated with TruSeq RNA Sample Prep Kit v2 Set A (Illumina). Briefly, poly(A) containing mRNA molecules were purified in two rounds using oligo(dT) attached magnetic beads from 1 μg of total RNA. After chemical fragmentation, mRNA fragments were reverse-transcribed and converted into double-stranded cDNA molecules. Following end-repair and dA-tailing, paired-end sequencing adaptors were ligated to the ends of the cDNA fragments using TruSeq PE Cluster Kit v3-cBot-HS (Illumina).

2.4. Deep Sequencing and Sequence Analysis. Libraries were sequenced using TruSeq SBS Kit v3-HS (Illumina) on an Illumina Hiseq 2000 machine at the Max Planck Institute for Molecular Genetics, Berlin. More than 10^8 100 nt paired-end reads were obtained from each sample and after quality assessment with package FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), the fastq files containing these reads were mapped to the mouse genome (build GRCm38 from *Mus musculus* C57BL/6J strain) together with the VACV WR genome (Genebank, AY243312.1) using Tophat v2.0.4 with default parameters [19]. Only those reads aligned against mouse genome were considered in a differential gene expression analysis with Cuffdiff (Cufflinks v2.1.0 software [19]). Since biological duplicates of samples from untreated cells were available, all comparisons were performed against this sample using the default mode of Cuffdiff, which is the most suitable for our kind of data. Pathway analysis of the significantly differentially expressed genes detected was performed using Ingenuity Pathway Analysis (IPA) software. Creation of proportional Venn diagrams and gene expression heatmaps were generated with the R "VennDiagram v1.6.9" and "Gplots" packages, respectively. The raw RNA-seq data has been deposited at the European Nucleotide Archive (ENA) under the project number PRJEB15047.

2.5. mRNA Expression by Real-Time-PCR (RT-PCR). To evaluate the expression levels of selected genes by RT-PCR, 1 μg of DNA-free total RNA isolated from L929 cells (three biological replicates per condition) was used for first strand cDNA synthesis with iScript cDNA Synthesis (BioRad) using oligo(dT) and random primers. Quantitative polymerase chain reaction (qPCR) analysis was performed using Fast SYBR Green PCR Master Mix (Applied Biosystem) with three technical replicates for each biological replicate, according to the manufacturer's recommendation in an ABI 7900 HT system (Applied Biosystem). Gene-specific qPCR primers were designed using primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and described in Table S1 in Supplementary Material available online at https://doi.org/10.1155/2017/5157626. Amplification was real-time-monitored and allowed to proceed in the exponential phase, until fluorescent signal reached a significant value (Ct). The fold change was determined using the $2^{-\Delta\Delta C(t)}$ method [20].

3. Results

3.1. The Type I IFN Cellular Response Is Inhibited in the Presence of the VACV B18 Protein. To characterize the inhibitory role of the VACV type I IFN binding protein B18 on IFN signalling we analyzed the RNA-seq profile of mouse L929 cells incubated with recombinant B18 before and after IFN treatment. We first determined the effect of type I IFN on global cellular gene expression and performed high-throughput RNA sequencing on total RNA obtained from cells mock-treated or treated with 50 units/ml of IFN-α for 4 h. Under these conditions, we identified a set of 46 significantly differentially expressed genes (SDEGs) after IFN treatment when compared to mock-treated cells (Table S2). Most of them (42 genes) were found to be upregulated in response to IFN while only 4 genes were downregulated. This set of IFN-stimulated genes (ISGs) contained several genes with previously known direct antiviral activity, such as APOL9, BST2 (Tetherin), DDX58 (RIG-1), EIF2AK2 (PKR), IFITM3, ISG15, MX2, OAS-1, PARP12, or TRIM. We also identified some ISGs involved in the positive regulation of IFN production such as IRF9, STAT1, STAT2, TRIM21, or TRIM30 and others encoding immunomodulatory molecules such as IL15, H2-Q1 (HLA-B), or UBC. We considered this our high-confidence gene set and performed a pathway enrichment analysis that mainly identified IFN related canonical pathways as statistically significant enriched (Figure 1(c)), indicating that L929 cells used in this study exhibited an appropriate biological response to IFN.

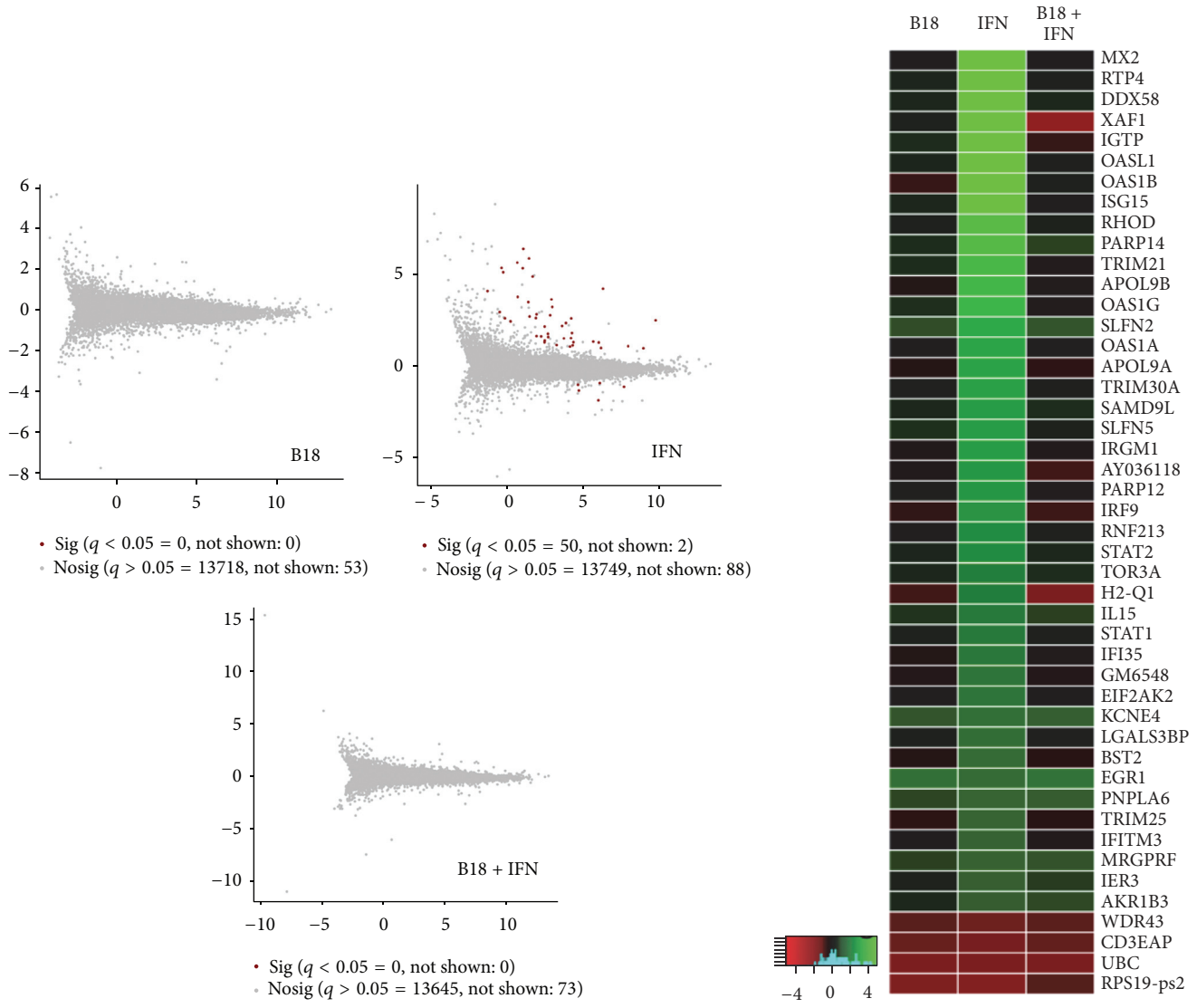
However, when cells were incubated with 0.45 μg/ml of recombinant protein B18 2 h before IFN addition we could not detect any significant change in cellular gene expression, indicating that the IFN-induced cell response was efficiently prevented by the addition of B18 (Figure 1). At the same time, it was confirmed that this concentration of B18 effectively protected against the antiviral effects of IFN (50 U/ml) using Vesicular stomatitis virus infection in HeLa cells (data not shown).

B18 is secreted from VACV-infected cells and has been previously shown to interact with GAGs at the surface of uninfected neighbouring cells to exert its inhibitory function [16]. This ability of B18 opens up the possibility of triggering additional signalling cascades after binding to GAGs on the cell surface. To test this possibility, cells were incubated for 4 h with the same amount of recombinant B18 used previously but in the absence of IFN. Importantly, under these conditions, no significant changes in the gene expression profile could be observed when compared to mock-treated cells, indicating no activation of host gene expression is triggered after the addition of B18 to cells (Figure 1).

To confirm these results, we selected three of the genes upregulated after IFN addition from the RNA-seq data (APOL9, IRF9, and OAS-1), together with other three genes whose expression was unaffected (DBF4, GAPDH, and MPRL2), and determined by RT-qPCR their expression levels. As expected, we found significant increased gene expression for APOL9, IRF9, and OAS-1 after IFN induction, and, concordant with the results from RNA-seq, the addition of B18 prior to IFN prevented this upregulation, keeping their expression values similar to those found in untreated cells (Figure 2). Moreover, DBF4, GAPDH, and MPRL2 expression determined by RT-qPCR remained unaffected after IFN induction or B18 incubation, as seen in the RNA-seq data (Figure 2).

3.2. VACV-Induced Changes on Cellular Gene Expression Profile. Searching for the initial response to VACV infection, we first explored by RNA-seq the transcriptomes of cells infected with UV-inactivated VACV and compared it with mock-treated cells. After alignment, only 0.17% of total reads matched the viral genome (Table S3), mostly corresponding to early VACV genes according to the temporal expression of VACV ORFs previously defined [21]. Under these conditions, we could identify changes in the expression of a modest set of 53 cellular genes (Table S4). Among these, the upregulation of some genes controlled by the NF-κB complex such as CCL5 (RANTES), H2-Q1 (HLA-B), the protein phosphatase DUSP5 involved in negative regulation of MAP kinases, the transcription factor FOSB, or SERPINE1 and also the downregulation of the macrophage migration inhibitory factor (MIF), CXCL1, ABCG1, SOD3, and negative regulator of NF-κB TRIB3 could represent the initial response to virus infection in the absence of viral genome replication. However the absence of type I IFN or IFN effectors should be noted.

By contrast, at 4 hpi with replication competent VACV, around 30% of total reads matched the virus genome, and a total of 2228 cellular genes were significantly differentially expressed compared to uninfected cells, 887 upregulated and 1341 downregulated (Table 1). In order to gain a comprehensive understanding of the transcriptomic changes induced by VACV infection we evaluated pathway enrichment by these SDEGs using Ingenuity Pathway Analysis (IPA) software. At this time postinfection the analysis identified severe alterations in cellular energy metabolism, since tricarboxylic acid (TCA) cycle, mitochondrial dysfunction, glycolysis, or oxidative phosphorylation represented the most significantly



	Canonical pathways	−log(p value)	Ratio	Molecules
(1)	Interferon signaling	7.87	0.139	OAS1, IFI35, STAT2, IRF9, STAT1
(2)	Activation of IRF by cytosolic pattern recognition receptors	6.48	0.067	DDX58, STAT2, IRF9, STAT1, ISG15
(3)	Role of pattern recognition receptors in Recognition of bacteria and viruses	4.15	0.037	OAS1, DDX58, Oas1b, EIF2AK2
(4)	Role of RIG1-like receptors in antiviral innate immunity	2.33	0.041	DDX58, TRIM25
(5)	Communication between innate and adaptive immune cells	1.73	0.018	HLA-B, IL15

(c)

Figure 1: Effect of B18 on type I IFN response. L929 cells were incubated with recombinant B18 protein (B18), with mouse IFNα (IFN), or with recombinant B18 and then mouse IFNα (B18 + IFN), and analyzed by RNA-seq. (a) Corresponding M/A plots representing expression of all cellular genes. M value (log 2 fold change) from each transcript between untreated and indicated sample cells is plotted against A (overall average log expression level) of each untreated and indicated pair. Red dots indicate SDEGs when compared to untreated cells after differential expression analysis. (b) Heatmap of SDEGs identified after IFN treatment. The heatmap displays the fold change expression (log 2) in the indicated samples relative to results for untreated cells. The colour scale is shown at the bottom of the heatmap. (c) Enriched canonical pathways after IPA analysis with SDEGs identified after IFN induction.

Table 1: Alignment of Illumina reads and number of differentially expressed genes from infected cells with the indicated viruses.

	PLWUV VACV		VACV 4 hpi		VACV 9 hpi		VACVΔB18 4 hpi		VACVΔB18 9 hpi	
Total reads aligned[a]	150.810.958		117.443.318		181.794.658		139.017.860		155.220.273	
Viral reads[b]	260.091 (0.17%)		37.708.314 (32.1%)		144.727.059 (79.6%)		25.497.885 (18.4%)		90.326.955 (58.2%)	
Cellular SDEGs	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
	18	24	887	1341	2398	3753	660	1013	1238	2309

[a] Aligned to either mouse or VACV Western Reserve genomes.
[b] Aligned exclusively to VACV Western Reserve genome.

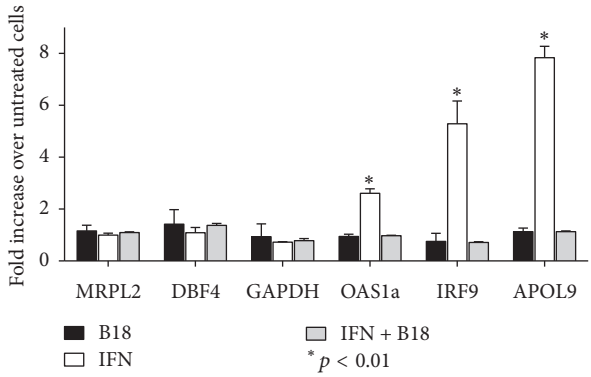


Figure 2: Confirmation of RNA-seq data by RT-qPCR after IFN induction. Gene expression for the indicated genes was assessed by RT-qPCR from L929 cells after incubation with B18, 50 units/ml IFNα, or IFN together with B18. β-Actin gene was used as a reference to normalize the data. Expression values are shown as fold change compared to untreated cells (mean ± SEM and significant differences are displayed).

enriched pathways (Figure 3(a)). Some of these pathways were predicted to be inhibited, indicating that infection was suppressing levels of a broad variety of proteins involved in energy metabolism early in infection. Signalling related to cell proliferation and differentiation was also found to be clearly affected during VACV infection, and examples were ERK/MAPK or PI3K/AKT signalling pathways that were modified. Differential expression of pathways specifically associated with cell-cycle arrest, such as G1/S and G2/M DNA damage checkpoints, p53 signalling, or ATM signalling, were also enriched following infection. Other significantly enriched pathways, such as actin signalling, Rac signalling, and integrin signalling, were related to cell migration and are consistent with the previously described VACV-induced cell motility during infection [22].

Most of these enriched pathways altered at 4 hpi were also found to be modified later in infection (at 9 hpi, Figure 3(b)), showing higher p values. However, at 9 hpi the analysis detected a striking overrepresentation of cellular genes involved in the modulation of protein translation. We observed the downregulation of 165 genes encoding ribosomal proteins and 45 encoding translation initiation factors. As a consequence, the most significantly enriched pathways identified at this time postinfection included EIF2 signalling, and regulation of EIF4 and p7056K and mTOR signalling (Figure 3).

3.3. The Absence of B18 during VACV Infection Does Not Markedly Alter the Cellular Gene Expression Profile. Previous analysis revealed the absence of IFN related pathways among enriched pathways altered after VACV infection, suggesting the viral downmodulation of type I IFN based host responses. Consistent with this, some of the ISGs with previously known antiviral activity, such as APOL9A, APOL9B, OAS1a, or OAS1g, exhibited lower expression levels in VACV-infected cells at 4 hpi as compared to mock-infected cells. We next evaluated the impact of B18 absence on cell host response during infection by using a VACV deletion mutant lacking expression (VACVΔB18). L929 cells were infected with VACVΔB18 and the gene expression profile was determined at 4 and 9 hpi and then compared to mock-infected cells. As shown in Table 1, 20% and 60% of the total sequencing reads corresponded to viral genes at 4 and 9 hpi, respectively. At 4 h after VACVΔB18 infection, a total of 1973 cellular SDEGs were identified when compared to mock-infected cells and the corresponding pathway enrichment analysis with these genes revealed that although 188 SDEGs were found exclusively differentially expressed during VACVΔB18 infection, most of these changes in gene expression were similar to those induced at the same times during wild type VACV infection, and no additional pathways were found among the 200 most significant enriched pathways (Figure 4).

3.4. Inhibition of the ISG Signature during VACV Infection Is Not Exclusively Dependent on B18. We also analyzed the IFN-mediated innate immune response after IFN treatment of infected cells. To this end, the expression levels of the ISGs previously identified after IFN treatment were determined by RNA-seq under various conditions. L929 cells were either (i) infected with wild type VACV and treated or not with IFN at 5 hpi, once the IFN inhibitor B18 had been produced and secreted; (ii) infected with VACVΔB18 and then treated or not with IFN (in the absence of B18); or (iii) infected with VACVΔB18 and supplemented with recombinant B18 before IFN addition. In all cases, total RNA was isolated at 9 hpi (4 h after IFN addition) and processed as indicated before. The results showed that addition of IFN to VACV-infected cells did not result in a clear activation pattern of the ISGs analyzed. We did not observe any difference in the ISG profile in cells infected with wild type VACV in the absence or presence of IFN, most likely due to the blocking action of secreted B18 to prevent IFN engagement with IFN cellular receptors. Surprisingly, even in cells infected with VACVΔB18, not producing B18, the addition of IFN did not

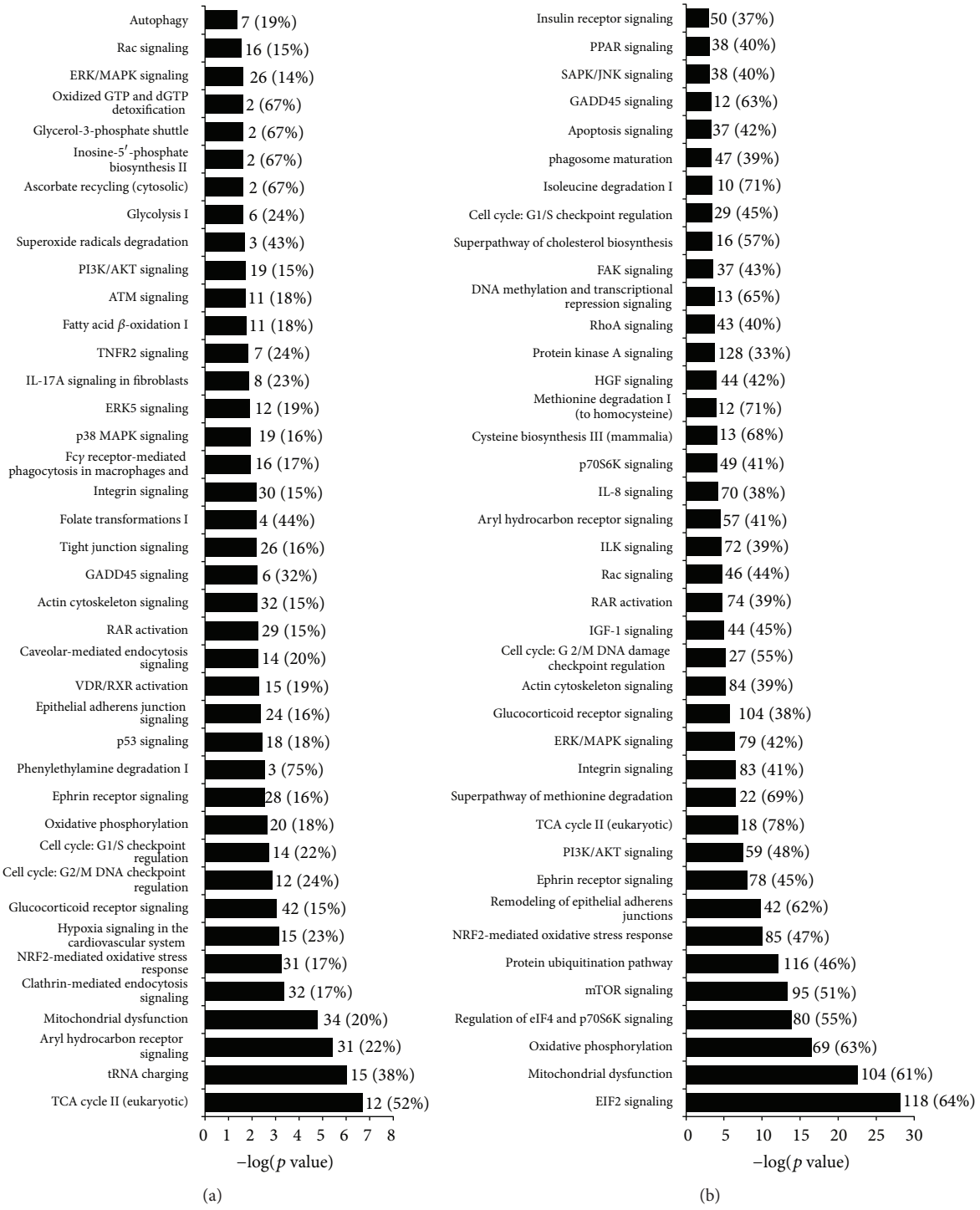


Figure 3: IPA analysis of differentially expressed host genes in VACV-infected cells. The list of SDEGs identified after VACV infection was used in a pathway enrichment analysis with IPA software. The 40 most significant pathways identified at 4 hpi (a) and 9 hpi (b) after VACV infection are shown. The x-axis represents the p value, indicating the significance of enrichment for the corresponding gene set. The values are plotted in a negative $\log_{10}$ scale.

result in an evident IFN based response, and the expression levels of the ISGs analyzed were similar to those found in VACVΔB18-infected cells in the presence of recombinant B18 protein and wild type VACV-infected cells (Figure 5).

In an independent assay, with additional RNA samples, we could confirm these results by RT-qPCR in cells infected with wild type VACV or VACVΔB18 in the same conditions described above. We first verified the expression of

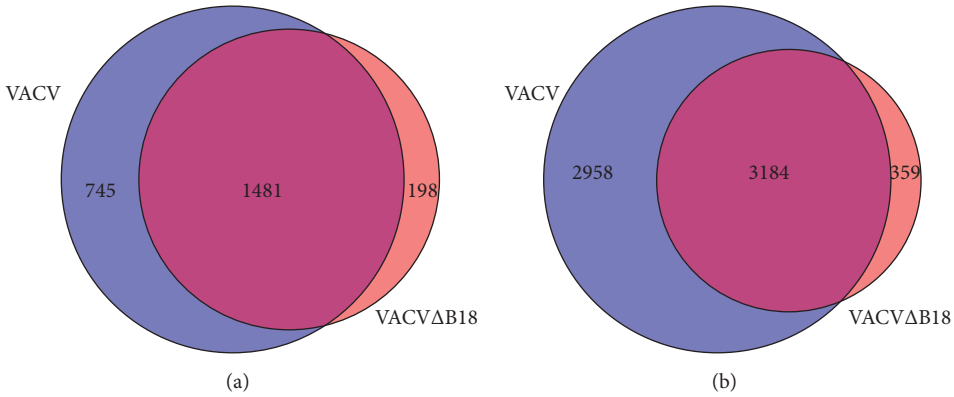


Figure 4: Effect of B18 absence on host gene expression during VACV infection. Venn diagrams showing the number of overlapped transcripts corresponding to cellular genes differentially expressed between VACV- and VACVΔB18-infected cells at 4 hpi (a) and 9 hpi (b) are displayed.

the viral genes WR092 and WR127 in all infected cultures and observed increasing expression values from 4 to 9 hpi, independently of the addition of IFN. On the contrary, but in concordance with results from the RNA-seq, the expression values of the ISGs determined by RT-qPCR (APOL9, IRF9, and OAS1a) during wild type VACV or VACVΔB18 infections, and independently of the addition of IFN, were similar in all cases to those detected in nontreated cells (Figure 6). Finally, no significant modification of cellular GAPDH expression levels, determined by RT-qPCR, was observed at 4 h after infection while a slight decrease was observed at 9 h during wild type VACV or VACVΔB18 infection in either the absence or presence of IFN.

4. Discussion

The secreted type I IFN binding protein B18 from VACV represents a unique strategy employed by poxviruses to evade the host IFN response. Its important contribution to the virulence of VACV and ectromelia virus, a related mouse-specific virus that also encodes a B18 orthologue, has been demonstrated in mouse models of infection [13, 23]. This anti-IFN activity has also been identified in the highly virulent variola virus and monkeypox virus [17]. In this report we have addressed the ability of the secreted type I IFN binding protein to modulate the expression of host genes regulated by IFN, using an RNA-seq approach to monitor the global expression of host and viral genes.

First, we evaluated the impact of type I IFN on the gene expression profile, required to induce an antiviral state and protect cells from infection. In the case of L929 mouse fibroblasts, we found the expression of 46 genes affected by the addition of IFN-α. Consistent with previous results demonstrating the ability of B18 to block IFN effects [13–16], the modulation of host gene expression by IFN could be efficiently prevented by the action of B18.

Using the same RNA sequencing approach, the incubation of cells with purified B18 protein did not cause any significant change in gene expression, suggesting that no cell signalling is triggered by B18. This result is of particular relevance since, after secretion from infected cells, B18 interacts with GAGs at the surface of infected and adjacent uninfected cells [14, 16], and GAGs have been shown to regulate multiple signalling pathways. This is the case of some growth factors, such as fibroblast, hepatocyte, or vascular endothelial growth factors [24–26], where the participation of GAGs is essential for receptor-ligand engagement and the resulting signalling. In contrast, our results clearly indicate that the interaction of B18 with GAGs at the surface of L929 cells does not trigger any detectable cell signalling leading to changes in host gene expression. Consistent with our results, it has been reported that addition of purified recombinant B18 to primary mouse plasmacytoid cells does not induce type I IFN production, whereas these cells were able to produce type I IFN in response to TLR ligands [27].

In this report we have determined the cellular transcriptome profile to investigate the changes in host expression during VACV infection. The host reaction to VACV seems to start immediately after infection, as deduced from the set of genes differentially expressed 4 h after infection with UV-inactivated VACV. Among these, we found some NF-κB regulated genes such as the proinflammatory chemokine RANTES/CCL5 gene, genes involved in the regulation of MAPK activity or the downregulation of antigen presentation related gene H2Q1, among others. It was somehow surprising that we did not detect more transcriptional activation in cells incubated with UV-inactivated virus, which should be able to attach and enter the cell. This may suggest that PRRs that activate cells in response to VACV infection detect mainly the viral genome that is being transcribed or replicated, rather than the small amount of virus particles that enter initially the cell (with viral proteins and DNA). Also, the incoming virus particle contains VH1, a phosphatase known to inhibit STAT1 and STAT2 activation, and may also prevent IFN responses in the absence of virus replication, as it was initially described [8]. Previous reports described the ability of inactivated VACV WR and monkeypox virus to induce the synthesis of IFN or IFN-inducible genes in plasmacytoid dendritic cells and macrophages, respectively [27, 28], but we could not detect the activation of these genes in L929 cells. Two factors could contribute to explaining these differences: the earlier timepoints analyzed compared with the previous

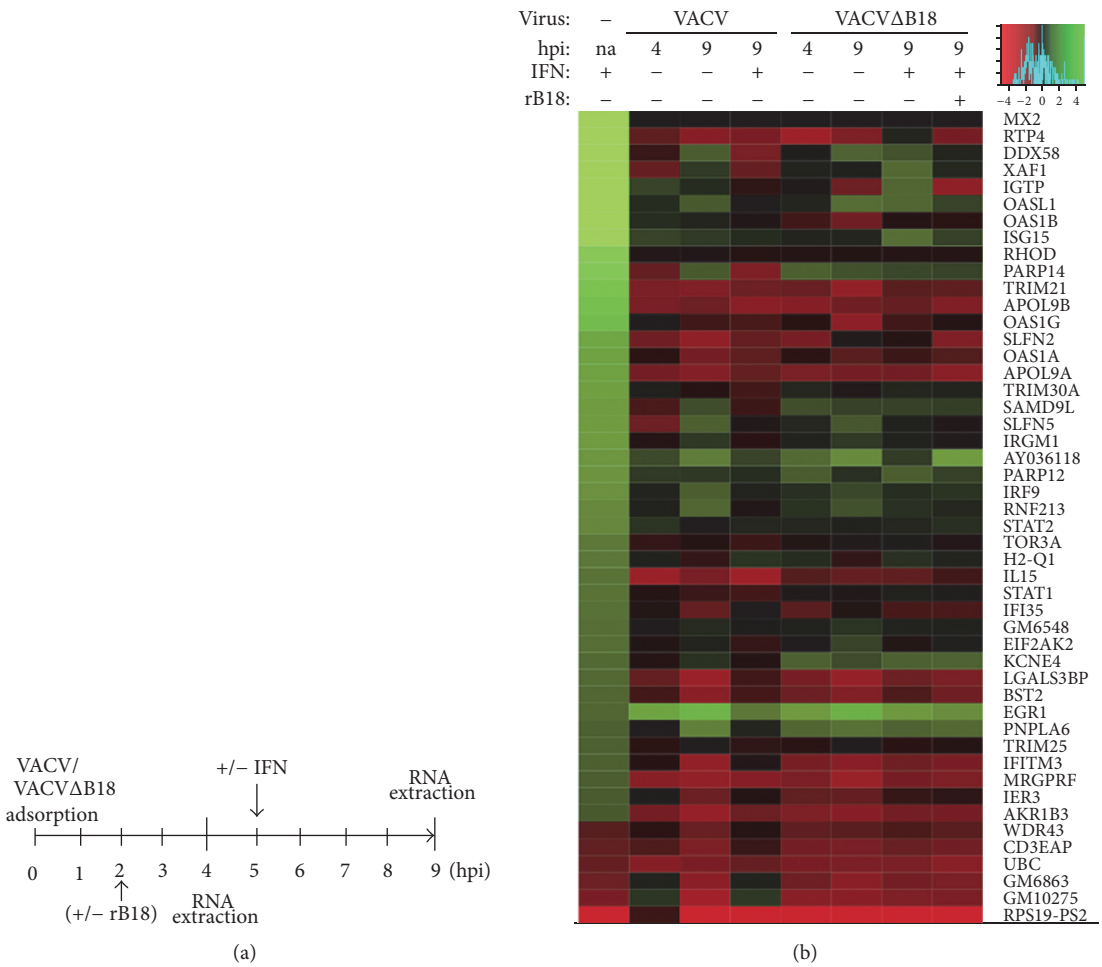


Figure 5: ISGs expression during VACV infection. (a) Diagram showing the experimental conditions to obtain RNA samples from infected cells. (b) Heatmap shows the expression levels of the ISGs previously identified relative to untreated cells, where indicated recombinant B18 protein (rB18) and/or mouse IFNα were/was added to cells at 2 and 5 hpi, respectively.

studies or the fact that the levels of IFN production are cell type dependent. Plasmacytoid dendritic cells are considered to be the professional type I IFN producing cells after viral infections [29, 30] and secrete much more IFN-α than other cell types.

By contrast, a drastic change in the host gene expression profile occurred after 4 h of infection with replication competent VACV, mainly affecting biological functions related to metabolism, cell death and survival, cell development and proliferation, and cell movement. Previous studies with HeLa cells using microarrays or deep RNA sequencing showed a general decrease in the cellular mRNAs upon VACV infection [21, 31]. In our study, as previously reported by Yang et al. [21], we found that the proportion of VACV mRNA was approximately 30% of the total mRNA, and more than 50% of modified cellular genes were downregulated at 4 hpi, which is indicative of the virus-induced degradation of cellular mRNA that precedes host translation shutoff [32]. This effect was even more pronounced at 9 hpi. Since the aim of our study was to analyze the modulation of type I IFN responses by B18, we selected the times postinfection that allowed the synthesis and secretion into the supernatant of an effective amount of the B18 protein, which is produced at early times of infection. Under these conditions, we focused on type I IFN responses upon VACV infection.

Our RNA-seq data from VACV-infected samples is in concordance with previous reports showing that, unlike the highly attenuated modified VACV Ankara (MVA) strain, virulent VACV WR infection of mice or dendritic cell cultures did not raise IFN-α responses. It is also known that the lack of a functional *B18R* gene and other IFN inhibitors in MVA allows the development of an IFN based host response during infection [33]. However, our data indicated that the infection with VACVΔB18, lacking expression of the secreted type I IFN inhibitor, equally failed to raise an effective IFN host response and VACVΔB18 infection proceeded similarly to VACV infection in L929 cells. This result corroborates the existence of additional viral mechanisms to inhibit the induction of type I IFN responses, as previously indicated by *Waibler and cols* during VACV infection of pDCs [27]. While B18 remains as the only identified secreted type I IFN inhibitor, during the last years diverse VACV genes have been

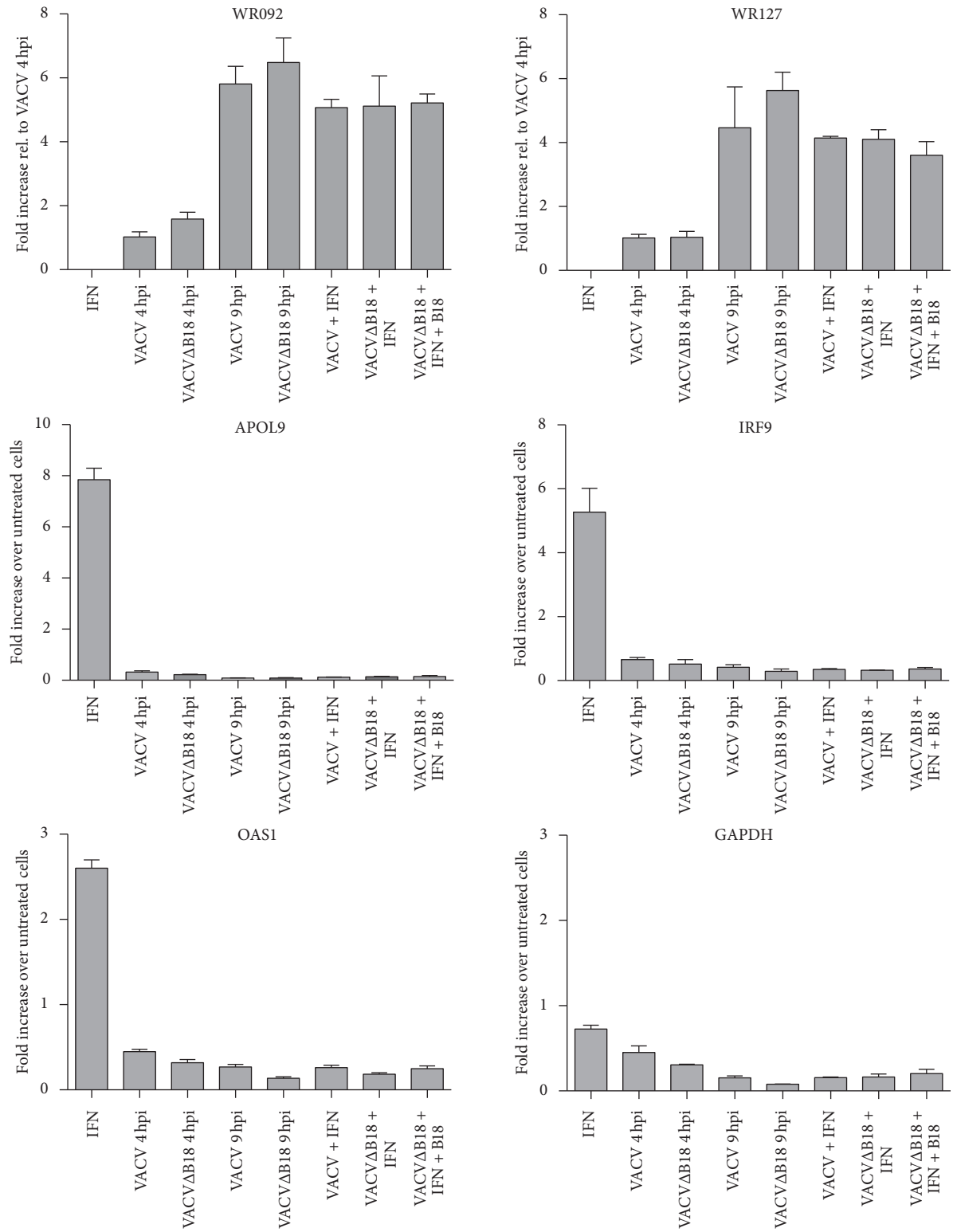


Figure 6: Confirmation of RNA-seq data by RT-qPCR during VACV infection. Gene expression for the indicated genes was assessed by RT-qPCR from VACV- or VACVΔB18-infected L929 cells at 4 hpi and 9 hpi. IFN was added to infected cells at 5 hpi where indicated. Expression levels of WR092 and WR127 VACV genes were also determined to monitor the progress of infection and relativized to VACV 4 hpi expression values. Mean from 3 biological replicates ± SEM and significant differences are displayed.

shown to have a direct role in the inhibition of the IFN production or the inhibition of the IFN signal transduction that takes place after type I IFN binding to cellular IFNAR [2]. Our results showed the lack of a functional IFN response during VACV infection in the absence of B18, and even after the addition of exogenous IFN-α, indicating that the IFN signalling downstream of IFNAR is impaired after VACV infection. We speculate that the virion associated phosphatase VH1, which dephosphorylates STAT1 and STAT2 to block downstream IFN-α signalling [8, 9], may contribute, together with other VACV genes, to explaining this lack of IFN responses during VACV infection in the absence of B18 function. In the cellular experimental system used here all cells were initially infected with VACV and hence the inhibition of the IFN response by B18 cannot be appreciated. The effect of B18 on virus replication in cell cultures treated with IFN is evident under other circumstances, such as when IFN is added a few hours after infection, as was illustrated in a previous report [14]. Deletion of the type I IFN binding protein in the VACV strain NYVAC has been reported to trigger the activation of IRF3, IRF7, and STAT1 and to increase the production of ISGs in human monocytes, in a transcriptomic analysis using microarrays [34]. The reasons for the different results reported in the previous report may be due to a different response in human monocytes or to the use of a highly attenuated VACV strain lacking many immunomodulatory genes, such as *C4L*, *N1L*, or *N2L*, which have been implicated in the modulation of intracellular signalling events [35–37]. Also, the recombinant viruses used in the NYVAC transcriptional studies have not been controlled for the potential inadvertent selection of mutations during the generation of the recombinant viruses, through the construction of revertant viruses or sequencing of the complete viral genomes, and thus the presence of additional mutations in other genes that may influence the reported results cannot be formally ruled out [38, 39].

The contribution of the secreted type I IFN binding protein to virus virulence and immune evasion becomes evident in mouse models of VACV and ectromelia virus infection, where mutant viruses show an attenuated phenotype that is dramatic in the mousepox model [13, 23]. In the animal host, the expression of a secreted IFN inhibitor is relevant to efficiently block the protective effects of IFN, which is produced in response to infection and is able to trigger IFN-mediated antiviral activities in neighbouring cells and restrict virus spread [23].

5. Conclusion

We have used RNA-seq to study by the modulation of the type I IFN response by VACV and the secreted type I IFN binding protein B18. This analysis identified cellular pathways modulated during VACV infection or induced by UV-inactivated virus particles. VACV B18 was a potent inhibitor of the type I IFN response, consistent with its ability to bind with high affinity IFN and to prevent its interaction with cellular IFNAR. VACVΔB18 inhibits the IFN response to an extent similar to that of wild type VACV, indicating that VACV encodes numerous mechanisms to block the IFN response and that the contribution of B18 to immune evasion is more evident in infected mice than in tissue culture. We also show that the interaction of B18 with cell surface GAGs does not trigger a specific host response leading to changes in host gene expression. The RNA-seq methodology allows the evaluation of the global gene expression in infected cells and the modulation of IFN responses by the VACV type I IFN binding protein. Future RNA-seq studies in VACV-infected mice may dissect better the ability of B18 to modulate the type I IFN-mediated response in different tissues.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Bruno Hernáez and Graciela Alonso equally contributed to this work.

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Review Article

The Role of Type III Interferons in Hepatitis C Virus Infection and Therapy

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The human interferon (IFN) response is a key innate immune mechanism to fight virus infection. IFNs are host-encoded secreted proteins, which induce IFN-stimulated genes (ISGs) with antiviral properties. Among the three classes of IFNs, type III IFNs, also called IFN lambdas (IFNLs), are an essential component of the innate immune response to hepatitis C virus (HCV). In particular, human polymorphisms in IFNL gene loci correlate with hepatitis C disease progression and with treatment response. To date, the underlying mechanisms remain mostly elusive; however it seems clear that viral infection of the liver induces IFNL responses. As IFNL receptors show a more restricted tissue expression than receptors for other classes of IFNs, IFNL treatment has reduced side effects compared to the classical type I IFN treatment. In HCV therapy, however, IFNL will likely not play an important role as highly effective direct acting antivirals (DAA) exist. Here, we will review our current knowledge on IFNL gene expression, protein properties, signaling, ISG induction, and its implications on HCV infection and treatment. Finally, we will discuss the lessons learnt from the HCV and IFNL field for virus infections beyond hepatitis C.

1. Type III Interferons

1.1. Interferon Lambda Genes and Proteins. Interferons (IFN) are innate cytokines, which interfere with virus infections. While type I IFNs were discovered in the 1950s, it was not until 2003 that the first type III IFNs, namely, IFN lambda 1 (IFNL1), lambda 2 (IFNL2), and IFN lambda 3 (IFNL3), were described [1, 2]. The most recent member of the type III IFNs, IFN lambda 4 (IFNL4), was discovered even ten years later [3, 4]. All four IFNLs are encoded on chromosome 9 in the 19q13.13 region. INFLs share their open reading frame structure with the interleukin-10 (IL-10) family of cytokines comprising five exons and four introns [5–7]. Therefore, they are also termed IL-29 (IFNL1), IL-28A (IFNL2), and IL-28B (IFNL3).

IFNL1 through IFNL3 have a high degree of sequence similarity with 72% to 96% amino acid conservation with IFNL2 and IFNL3 being most closely related. These findings suggest a common ancestor gene for all IFNLs [3]. IFNL4 expression is the consequence of a frameshift mutation and this gene product shares 27% to 29% sequence similarity with the other three IFNLs (Table 1 and Figure 1). IFNL1–3 proteins are roughly 22 kDa in size, while IFNL4 is slightly smaller with 20 kDa. They share an alpha helical bundle structure with type I and type II IFN family members. Significant differences occur in the side chains of IFNL1, IFNL2, and IFNL3 and amino acid differences at the receptor binding site likely contribute to the differences in IFNL responses as detailed below.

1.2. IFNL Expression. The expression of IFNL genes is tightly controlled and expression profiles of IFNL subtypes are ligand and tissue specific [8]. Typically, RNA virus infection and the concomitant exposure of cells to foreign RNA in cytoplasmic or endosomal compartments lead to IFNL induction. In particular, Sindbis virus, dengue virus, vesicular stomatitis virus, encephalomyocarditis virus [1, 2], respiratory syncytial virus [9, 10], influenza virus, Sendai virus [11, 12], and hepatitis C virus (HCV) [13–15] were shown to induce IFNLs in vitro and in vivo. In addition to RNA viruses, DNA viruses including cytomegalovirus and herpes simplex virus can induce IFNLs [16, 17]. While almost any cell type can express IFNLs, the most prominent producers

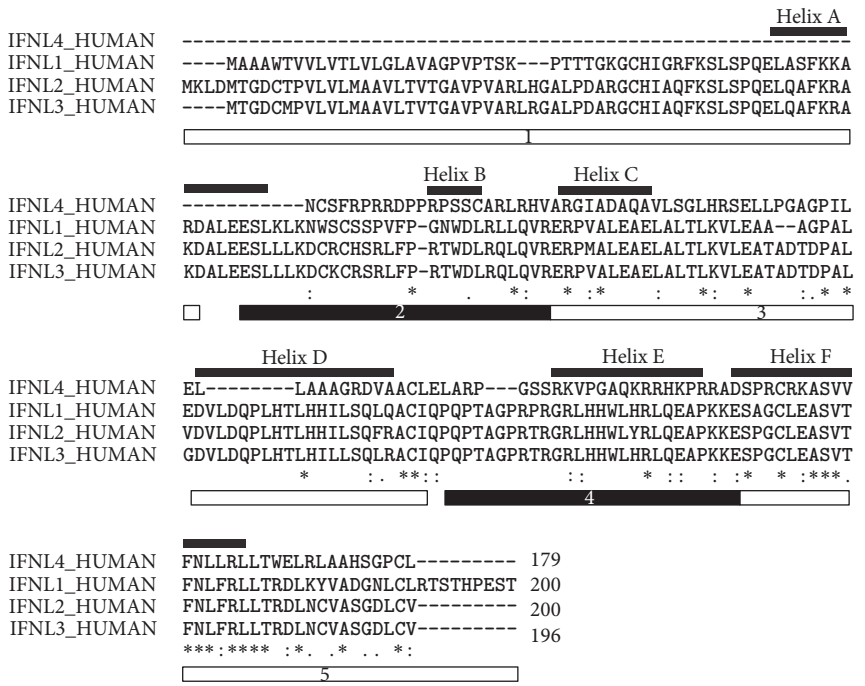

Figure 1: Sequence alignment and amino acid conservation of IFNLs. Clustal Omega (1.2.3) alignment [37] of IFNL proteins (IDs: Q8IU54, Q8IZJ0, Q8IZI9, and K9M1U5). Exons are indicated by the black and white boxes below the sequences. Positions of helices are indicated by the lines above the sequences. Identical amino acids are marked by an asterisk (∗); conserved amino acids by a colon (:); and semiconserved amino acids by a period (.).

of these antiviral cytokines are myeloid and plasmacytoid dendritic cells [8, 18–21]. Tissues with strong IFNL induction upon virus infection are the lung and the liver with a strong contribution of airway epithelial cells and hepatocytes [15, 22–29]. Limited data is available on the expression kinetics of IFNLs in different cell types. It seems, however, that IFNL expression onset and duration differ for the four subtypes. For instance, primary human hepatocytes (PHH) carrying the single nucleotide polymorphism (SNP) responsible for IFNL4 expression show an early and short IFNL4 expression (2 to 6 h after stimulation), while IFNL3 was detectable from 2 to 24 h after stimulation with a synthetic poly I:C RNA ligand [4]. Differences in positive or negative feedback mechanisms may explain the varying expression kinetics for IFNL subtypes. IFNL1 through IFNL3 are typically induced simultaneously and this is reflected by common transcription factors and binding sites in the promoter regions. Activator protein 1, IFN response factor 3 (IRF3), IRF7, and nuclear factor kappa beta (NF-kB) are thought to bind to the promoter of all INFL genes [11, 12, 30–36]. Additionally, Med23 seems to be a transcriptional coactivator [17]. Taken together, IFNLs are induced upon sensing of virus infection in particular after lung and liver infection.

1.3. The IFNL Receptor. The receptor for all four IFNLs is composed of two subunits, the alpha-subunit IFNLR1 encoded on chromosome 1 and the beta-subunit IL10RB encoded on chromosome 21 [40–44]. The former is specific for the IFNL receptor (IFNLR), while the latter is shared

Table 1: Amio acid conservation of IFNLs.

	IFNL1	IFNL2	IFNL3	IFNL4
IFNL1	100	72.77	73.82	27.59
IFNL2	72.77	100	95.92	26.89
IFNL3	73.82	95.92	100	28.57
IFNL4	27.59	26.89	28.57	100

Clustal percent identity matrix of IFNL1–4 using MUSCLE showing amino acid identities (%) (protein IDs: Q8IU54, Q8IZJ0, Q8IZI9, and K9M1U5).

with the type II cytokine receptors for IL-10, IL-22, and IL-26 [45]. Restricted expression of the IFNLR1 subunit leads to a tissue specific response to IFNLs. In particular tissues with high epithelial cell content like intestine, liver, and lung express IFNLR1 and respond to IFNLs [46]. Apart from primary human hepatocytes, hepatocellular carcinoma cell lines including Huh-7 and HepG2 cells respond to IFNL [14, 47]. In addition to the full length IFNLR1, a secreted form lacking exon VI has been described and may function as a decoy receptor dampening IFNL responses [2, 48, 49].

The IFNL ligand-receptor interface is comprised of helix A, loop AB, and helix F for IFNL and the N-terminal domain as well as the interdomain hinge region for the IFNLR. Van der Waals and hydrophobic forces determine the ligand-receptor interaction [40]. Amino acids critical for receptor binding differ between IFNL subtypes and this might lead to different ligand binding affinities as well as differences in the stability of the ligand-receptor complex [4, 50]. Additionally, mutations in the IFNL3 and IFNL1 receptor binding sites

have been described with the IFNL4 generating frameshift mutation being the best described [40, 41, 50]. The impact of these genetic variants is discussed in detail below. Taken together all four IFNL proteins share the same cell surface receptor, which is primarily expressed in intestine, lung, and liver tissue.

1.4. IFNLR Signaling. Signaling in response to IFNLs is initiated by dimerization of the two IFNLR subunits. Initial binding of IFNLs to IFNLR1 induces the recruitment of IL-10RB, leading to the activation of the receptor associated kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). Cross-tyrosine phosphorylation of the IFNLR subsequently recruits signal transducer and activator of transcriptions (STAT) 1 and 2 to the receptor platform. Phosphorylated STAT1 and STAT2 form a heterotrimer together with IFN regulatory factor 9 (IRF9). This trimeric complex, called IFN-stimulated gene factor 3 (ISGF3), translocates to the nucleus where it binds to the IFN regulated response element (ISRE) to drive IFN-stimulated gene (ISG) expression [51].

Antiviral effects of IFNLs are largely shared with type I IFNs. However, differences in receptor tissue expression and the kinetics of STAT pathway induction exist between the two IFN classes [52–54]. In Huh-7 cells, IFNL induces a slower and more sustained ISG response [55, 56]. Among the hundreds of ISGs induced by IFNLs and type I IFNs are ISG15, myxovirus (influenza virus) resistance 1 (MX1), $2'$-$5'$-oligoadenylate synthetase 1–3 (OAS-1–3), and protein kinase R (PKR). ISGs interfere with different stages of viral life cycles as reviewed in [57]. The anti-inflammatory ISGs USP18 and suppressor of cytokine signaling 1–3 (SOCS1–3), however, are specifically induced by IFNLs and not by type I IFNs [58]. Both proteins interfere with STAT signaling and therefore lead to desensitization to type I IFNs and IFNLs [59–61]. IFNL4 additionally induces expression of *rantes* and *fos* genes in hepatoma cells [4]. These genes are hallmarks of HCV-induced liver damage. Interestingly and in contrast to type I INFs, IFNLs are themselves ISGs as IFN stimulation of hepatoma cells induces their expression [11].

Although IFNL2 and IFNL3 have high sequence homology, they differ in their antiviral activity with IFNL3 displaying the strongest antiviral activity in a HepG2 challenge experiment with encephalomyocarditis virus [62]. This finding is in line with a strong ISG (MX1 and IRF9) induction by IFNL3 in hepatocytes [55]. IFNL4, in turn, displays antiviral activities which are comparable to IFNL3 as shown in reporter cells expressing the IFNLR and a luciferase gene under the control of the IFI6 promoter [3]. In conclusion, IFNLs signal through the JAK1/STAT pathway for ISG induction and the set of ISGs largely overlaps with that induced by type I IFNs.

2. Hepatitis C Virus

2.1. Molecular Virology of HCV. The hepatitis C virus belongs to the genus *Hepacivirus* in the Flaviviridae family. HCV is an enveloped virus with a single-stranded, positive-orientated RNA genome of 9.6 kbp length. According to genome sequence diversity HCV can be classified into seven genotypes and multiple subtypes [63]. The liver tropic virus enters hepatocytes in a multistep process involving several host cell proteins (as reviewed, e.g., in [64]). After pH-dependent fusion of the viral membrane with the endosomal membrane, the viral genome is released into the cytoplasm. There the positive-orientated RNA genome is directly translated into a single polyprotein, which is cleaved by viral and cellular proteases into 10 structural and nonstructural (NS) proteins. Replication and virus assembly occurs in endoplasmic reticulum- (ER-) associated membranous structures, called the membranous web (MW) (as reviewed, e.g., in [65]). HCV assembly, maturation, budding, and release occur in close contact with the cellular very low density lipoprotein synthesis pathway. Nascent HCV particles are released from the cells via the secretory pathway into the bloodstream or directly infect bystander cells (as reviewed, e.g., in [64]). A schematic overview of the HCV life cycle is depicted in Figure 2.

2.2. Pathogenesis and Treatment of Hepatitis C. Worldwide 92–149 million people, representing approximately 2% of the world's population, are chronically infected with HCV [66], one of the causative agents of viral hepatitis. HCV is a blood borne virus and transmission occurs parenterally, mainly by reusing injection material, insufficient sterilization of medical tools, or by transfusion of unscreened blood or blood products. As screening of blood products is a standard procedure nowadays in most countries, people who inject drugs have the highest risk of contracting hepatitis C. In fact, over 60% of injecting drug users are positive for HCV-antibodies [67].

Naturally HCV infects only humans, but chimpanzees can be experimentally infected. In both cases, HCV targets the liver, in particular hepatocytes. The narrow host range is determined by the presence or absence of certain host cell factors; proteins critically needed for HCV entry, like the cell surface receptors scavenger receptor BI (SRBI), CD81, claudin-1 (CLDN1), and occludin (OCLN) (as reviewed, e.g., in [68]) or molecules needed for viral replication, like microRNA 122, are expressed in hepatocytes. On the other hand, proteins suppressing HCV infection, like EWI-2wint, are absent in hepatocytes [69].

After acute infection, which is mainly asymptomatic, HCV establishes a lifelong, persistent intrahepatic infection in approximately 80% of the patients. Development of chronic hepatitis C (CHC) leads to progressing liver fibrosis and eventually cirrhosis (15–30% of CHC patients), which can cause liver failure or the development of hepatocellular carcinoma (2–4% of CHC induced cirrhosis patients per year) [70]. Consequently, HCV causes app. 700,000 deaths per year [70].

CHC was classically treated with recombinant PEG-IFN-alpha in combination with Ribavirin (RBV). The treatment duration was long (24–48 weeks) and wearing, with PEG-IFN-alpha being administered 3 times a week, severe side effects occurring frequently, and still only approximately half of the patients being cured. Since 2014 HCV therapy

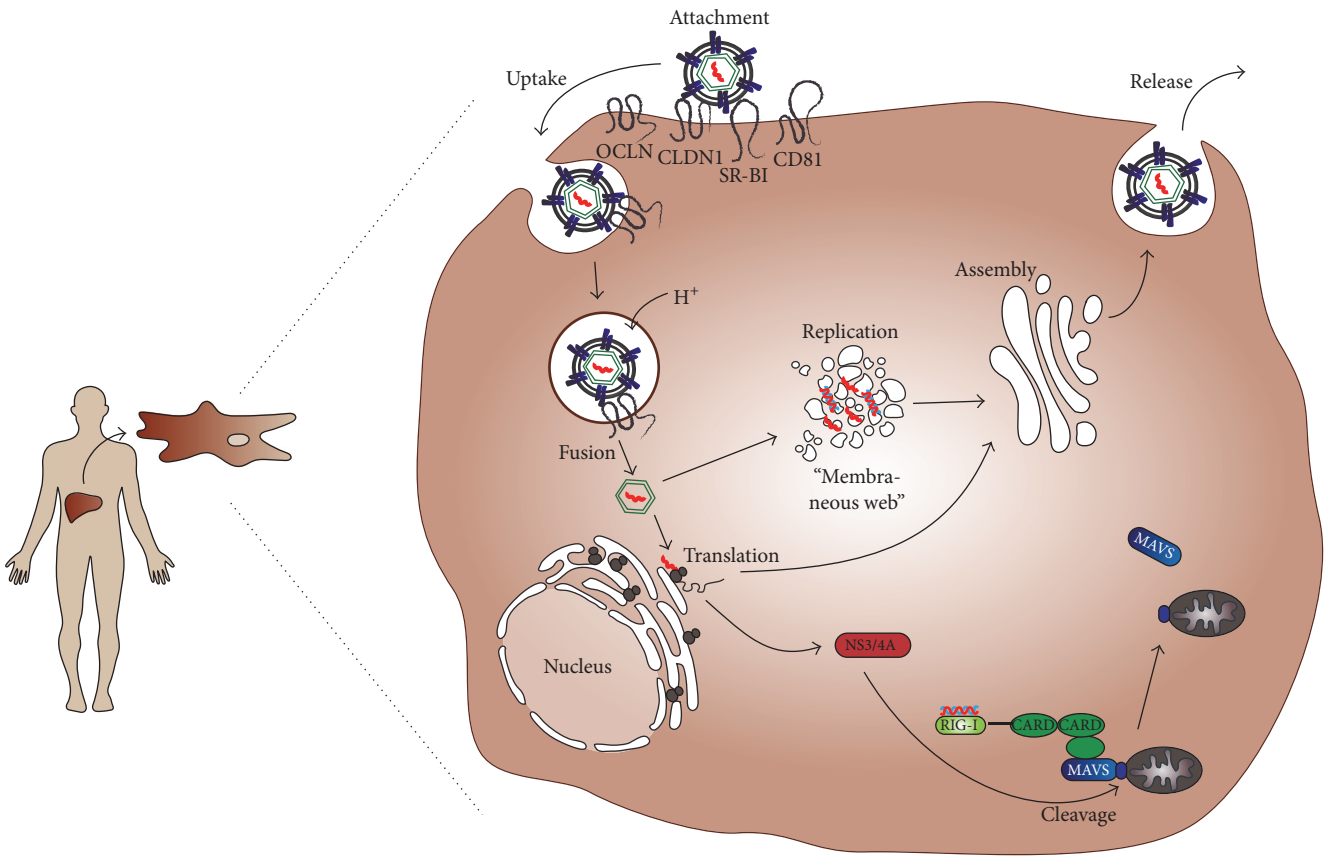

Figure 2: Hepatitis C virus life cycle and MAVS cleavage. Upon transmission, HCV enters hepatocytes in a multistep process involving the four host factors SR-BI, CD81, CLDN1, and OCLN. After uptake and pH-dependent fusion of viral and endosomal membranes, HCV releases its RNA genome into the cytosol of the host cell. Replication via double-stranded RNA intermediates takes place in the membranous web, consisting of ER-derived structures. HCV RNA is then translated into a precursor protein, which is cleaved into ten mature viral structural and nonstructural (NS) proteins. One of the latter, NS3/4A, can proteolytically cleave and by this inactivate MAVS, a RIG-I and MDA5 adaptor protein, which is important for mounting an innate immune response against HCV infection (see text and Figure 3 for details).

improved drastically, as several direct acting antivirals targeting HCV NS3/4A protease, NS5A, or NS5B RNA-polymerase were approved. These inhibitors, either alone or in combination with RBV, now heal over 90% of patients treated. Direct acting antivirals (DAAs) are more effective than PEG-IFN-alpha in eliminating HCV, but also treatment duration is shorter (minimum of 8 weeks), they can be administered orally, and adverse events are fewer. Despite advances in drug development, a vaccine against HCV is still not available. For more details we refer the reader elsewhere, for example [71, 72].

2.3. Innate Immunity to HCV. The innate immune response serves as the first line of defense against infections; pathogen associated molecular patterns (PAMPs) are recognized by extra- or intracellular pattern recognition receptors (PRRs), which triggers signaling cascades leading to the production of cytokines including interferons. The innate immune response to HCV is summarized in Figure 3.

In HCV infected cells, double-stranded (ds) RNA replication intermediates are generated and recognized as PAMPs by the cytosolic RNA sensor retinoic acid-induced gene I (RIG-I) [73] and melanoma differentiation-associated gene 5 (MDA5) [74, 75], both belonging to the family of RIG-I like receptors (RLRs). Sensing of HCV by RIG-I or MDA5 then leads to the oligomerization of the adaptor protein mitochondria antiviral signal (MAVS; also called CARDIF, VISA, IPS-1) into large signaling complexes [76].

Besides the cytosol, HCV dsRNA can also be present in extracellular, ER, or endosomal compartments. Extracellular dsRNA, maybe released from dying cells, can be taken up into uninfected neighboring cells by class A scavenger receptors [77]. After endocytosis, dsRNA is brought to the endosome, where it is bound by Toll-like receptor 3 (TLR3). Alternatively, TLR3 might engage HCV in autophagic vesicles, as HCV replicating cells display an enhanced amount of them [78]. Recognition of dsRNA by TLR3 activates TIR domain-containing adapter-inducing IFN-β (TRIF; also called TICAM-1) signaling.

MAVS and TRIF then trigger signaling cascades leading to the activation of different cytosolic kinases (IκB kinases (IKK) and TANK-binding kinase 1 (TBK1)), which in turn induce activation of the key transcription factors NF-κB and IRF3 [79, 80]. Upon activation, these proteins translocate to

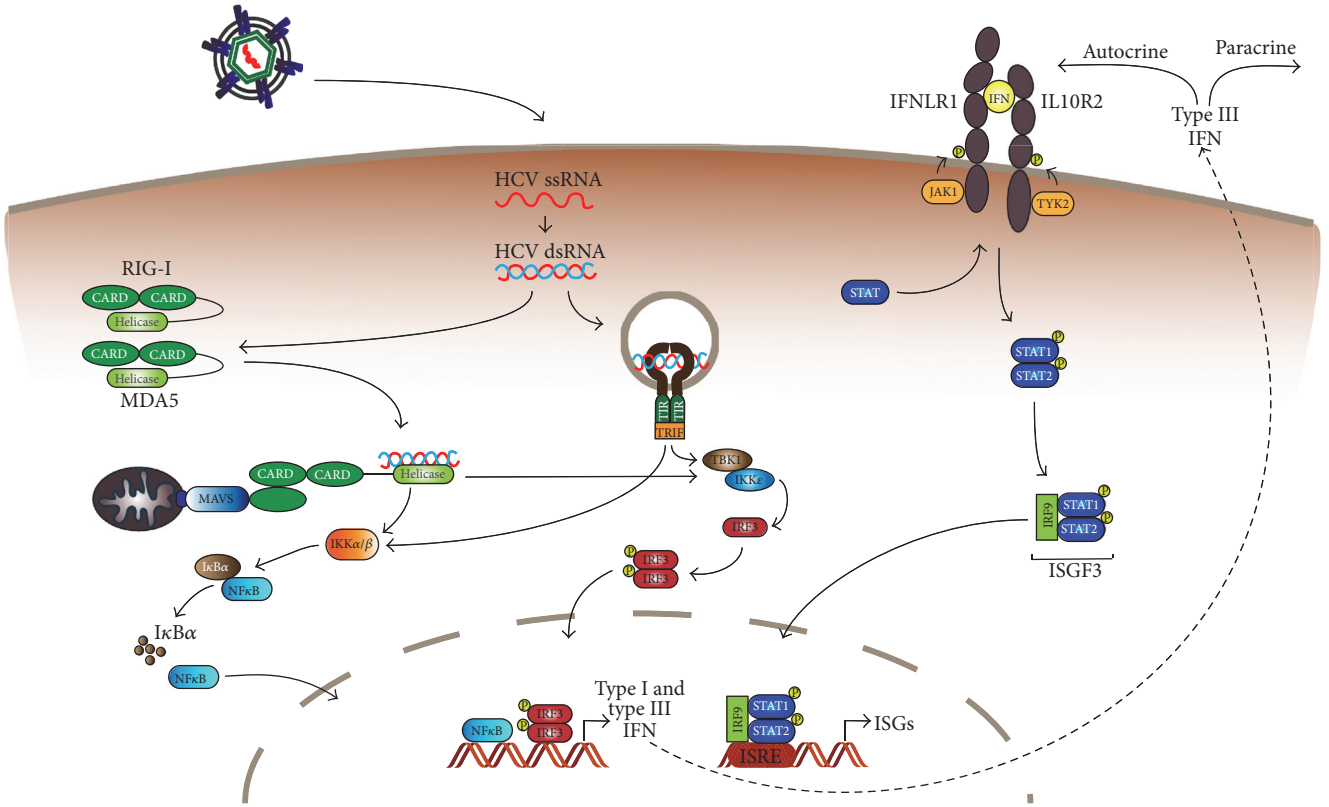

Figure 3: Innate immunity against HCV. In HCV infected cells dsRNA replication intermediates are recognized by the cytosolic RNA sensors RIG-I and MDA5, leading to the activation of MAVS. Endosomal dsRNA is recognized by TLR3, leading to the activation of TRIF. MAVS and TRIF signal via the kinases IKK and TBK1, resulting in translocation of the transcription factors NF-κB and IRF3 to the nucleus. Here they trigger the expression of type I and type III IFNs, which are secreted and bind to their receptors in an auto- or paracrine manner. Subsequently, the JAK/STAT pathway is activated, which ultimately initiates the expression of ISGs, generating an antiviral state.

the nucleus where they bind to promoter elements in type I and type III IFN genes. By this, inflammatory cytokine and IFN expression is initiated. Binding of secreted IFNs to their receptors in an autocrine or paracrine manner leads to the activation of the JAK/STAT pathway, as depicted in Figure 3. Ultimately, this triggers the expression of hundreds of IFN-stimulated genes ISGs, which generate an antiviral state limiting HCV replication.

During CHC, the innate immune response can only control HCV replication but not completely eliminate the virus. This is partially due to viral mechanisms counteracting the immune response: Briefly, HCV NS3/4A serine protease has been shown to inhibit IFR3 phosphorylation [81] by cleaving and inactivating the RIG-I adaptor protein MAVS [82–84] and the TLR3 adaptor protein TRIF [85]. Interestingly, recently discovered members of the genus Hepacivirus infecting nonhuman mammals carry an NS3/4A enzyme capable of cleaving not only their cognate host's MAVS, but also human MAVS [86]. This suggests that all yet studied hepaciviruses can antagonize the human antiviral innate immune response and that there is no barrier to zoonotic transmission at the level of innate immune interference.

3. IFN Lambda and HCV Infection

3.1. Induction and Role of IFN Lambda in HCV Infection

3.1.1. IFNL in CHC Patients. Humans chronically infected with HCV display increased IFNL expression. Specifically, Dolganiuc et al. showed that IFNL serum levels are higher in CHC patients than in HCV-negative with liver inflammation [87]. The authors observed elevated expression of the IFNLR in liver biopsies from CHC patients. Studying liver biopsies from infected patients also helped to confirm that there is an association between IFNL and ISG expression levels; namely, that IFNL expression leads to elevated ISG induction [88, 89]. In particular, a correlation between the activity of the IFNL4 protein and ISG induction has been discovered. Surprisingly, high IFNL4 and ISG levels negatively impacted the outcome of HCV infection and treatment (see Section 3.2) [90].

3.1.2. IFNL in Experimental Animal Models of Hepatitis C. The host immune response to acute HCV infection has been studied in experimentally infected chimpanzees and in genetically humanized mice. In the livers of chimpanzees

a strong host response can be detected, including the induction of type III IFN transcription and ISG expression [88, 91]. Especially IFNL1 mRNA and protein levels are elevated, correlating with ISG expression and viremia. However, there is no link between type III IFN expression in the liver or peripheral organs of infected chimpanzees and the outcome of the acute infection [91]. Consistently, in immunocompetent transgenic mice expressing the human HCV entry factors, HCV infection results in upregulation of several ISGs [92]. This is consistent with the observation that mouse-liver derived cells produce type I and III IFNs when transfected with HCV subgenomic RNA and this leads to abrogation of HCV replication [93]. Of note, current mouse models do not allow chronic HCV infection and since the ban on chimpanzee experimentation there are no immunocompetent animal models to study CHC. Recent efforts on establishing alternative nonhuman primate models for hepatitis C [94, 95] and on using rodent hepaciviruses as surrogate infectious agents to study CHC, might resolve this hurdle in the future [96, 97].

3.1.3. IFNL in In Vitro Models of Hepatitis C. In line with observations made in livers of infected chimpanzees HCV infection induces the expression of IFNL in primary human hepatocytes [88, 91]. Type III IFNs and ISGs are similarly inducted upon HCV infection of primary human fetal liver cells [98, 99]. Here the magnitude of induction differs from donor to donor but correlates with virus replication.

To study different aspects of the HCV life cycle, hepatoma cell lines are frequently used. Similar to HCV infection of primary cells, also the hepatoma cell line HepG2 induces IFNL transcription upon infection [74, 88]. Interestingly, Israelow et al. showed that IFNL induction attenuates HCV replication and that the IFNL response in HepG2-HFL cells stably replicating a HCV subgenome is blunted, probably due to MAVS cleavage by HCV NS3/4A [100]. With regard to IFNL4, Hong et al. found that endogenous IFNL4 transcription is only poorly induced upon stimulation with HCV in different hepatoma cell lines and PHH. Also no or reduced levels of secreted IFNL4 as compared to IFNL3 are detectable upon HCV infection [3]. The partial retention of IFNL4 in the cytoplasm, as observed in HepG2 cells and PHH in a different study, might explain this observation [101].

The clear correlation between IFNL induction and HCV attenuation observed in hepatoma cell lines does not reflect observations in CHC patients for several reasons. First, the complexity of the liver with contributions of Kupffer cells, liver sinusoidal endothelial cells, stellate cells, and infiltrating additional immune cells (reviewed in [102]) is obviously not mimicked by simple cell culture models. Second, transformed cell lines do not necessarily resemble primary hepatocytes. In fact, most hepatoma cell lines that can be infected with HCV do not mount a strong innate immune response [73]. Nevertheless hepatoma cell lines are regularly used to study the effect of exogenously added IFNL on HCV infection as they typically express all components of the IFNLR pathway. IFNL stimulation reduces levels of subgenomic or full length genomic HCV (+)RNA in Huh-7 cells in a dose dependent-manner [49, 103, 104]. These results were confirmed in several other hepatoma cell lines, including the Huh-7 derived Lunet hCD81 cells expressing a firefly luciferase gene or HepG2 cells expressing microRNA122 and CD81 [3].

Hepatoma cell lines and PHH have also been used to study how the IFNL subtypes differ in their ability to limit viral infections; IFNL3 and IFNL4 induce the same set of ISGs in PHH [105] and the two subtypes have the same antiviral activity against HCV in an overexpression setup in hepatoma cells. In summary, expression of specific IFNL subtypes is induced in PHH and some hepatoma cell lines upon infection with HCV, resulting in limiting virus production. However, the majority of hepatoma cell lines do not elicit a strong immune response and IFNL expression. Novel model systems including stem cell derived hepatocytes [106–108] and tissue engineering systems [109] might in the future allow to more faithfully mimic host responses to hepatotropic virus infection.

3.2. IFN Lambda Polymorphisms. After establishment of PEG-IFN-alpha and RBV as the standard of care treatment for hepatitis C [110], it became clear that patients of African ancestry had significantly lower cure rates than those of European ancestry during IFN-alpha/RBV treatment. In 2009, two genome-wide association studies discovered IFNL gene polymorphisms as the underlying genetic basis for the different IFN-alpha/RBV treatment responses as well as for different spontaneous clearance rates [111, 112]. This work spurred further investigations on IFNL gene SNPs and their role during HCV infection and treatment.

3.2.1. Role of IFNL Polymorphisms in Antiviral Therapy. Three major SNPs near the IFNL3 and IFNL4 genes correlate with HCV treatment response and are in high linkage disequilibrium [113, 114]. These polymorphisms are rs12979860(C/T) located 3 kb upstream of the IFNL3 gene [111, 112], rs8099917 (T/G) located between the IFNL2 and IFNL3 genes [42, 115], and rs368234815(TT/ΔG) (originally named ss469415590), which creates a frameshift upstream of the IFNL3 gene leading to generation of the new IFNL4 gene product [4, 38, 116]. For all three SNPs the first allelic variant is associated with a higher probability of sustained virological response to IFN-alpha/RBV treatment. The location of these three SNPs on chromosome 19 is schematically depicted in Figure 4.

Treatment response dependency on IFNL polymorphisms was demonstrated for several HCV genotypes and in chronic patients with genotype 4 the IFNL SNPs are the strongest predictors for response known to date [117]. In addition to the three above described SNPs, six additional SNPs in the IFNL locus have been described to strongly associate with sustained virological response after IFN-alpha/RBV treatment (rs8105790, rs11881222, rs8103142, rs28416813, rs4803219, and rs7248668) [115].

How the IFNL SNPs mechanistically influence treatment outcome is mostly unclear. Initially, it was suspected that the SNPs alter the transcriptional regulation of IFNL3 as they are located upstream of the IFNL3 coding sequence, where

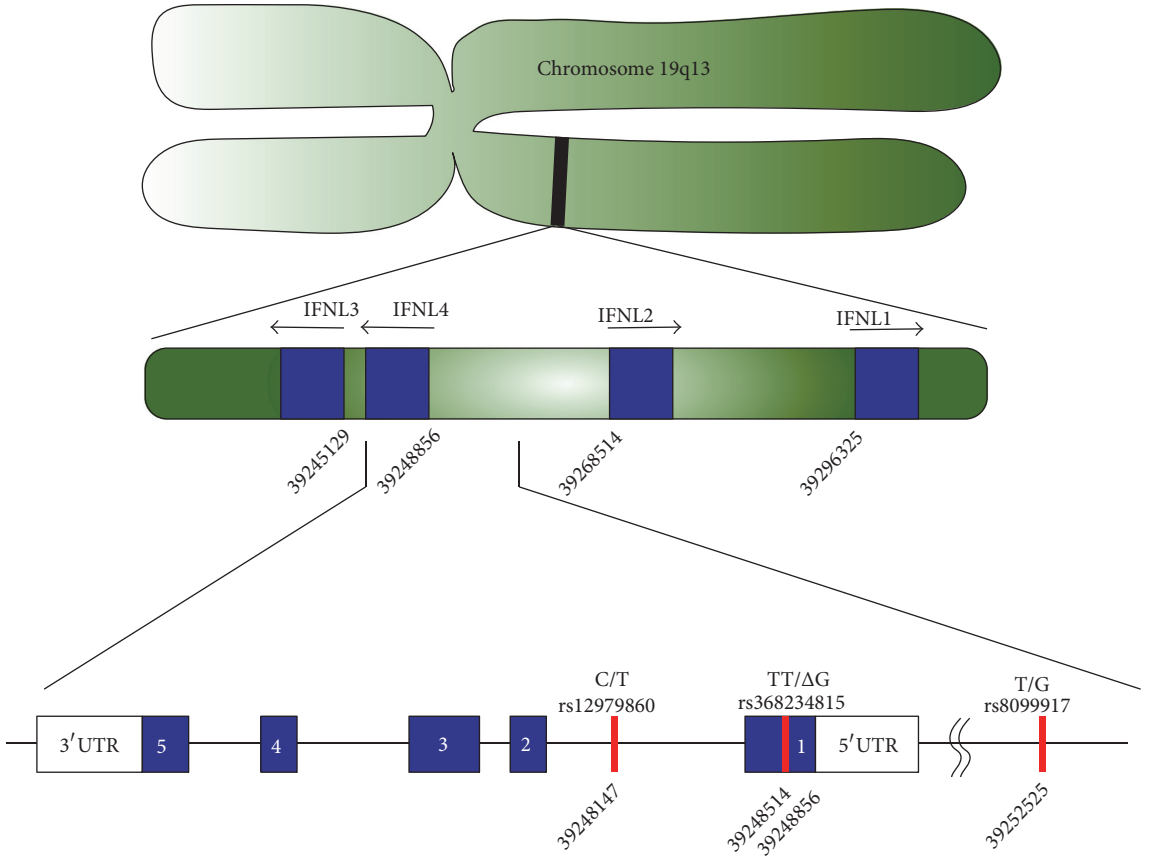

Figure 4: Schematic representation of the IFNL chromosome locus. Depicted is the position and orientation of the genes for IFNL1–4 (blue) on chromosome 19 q13 (green). The numbers below the genes display the nucleotide position of the first exon of each IFNL subtype. IFNL1-2 are located on the forward strand of the chromosome, while IFNL3-4 are located on the reverse strand. The genetic organization of IFNL4 is shown in more detail; it consists of five exons (blue) and 3′/5′ untranslated regions (UTR). Single nucleotide polymorphisms rs12979860, rs368234815, and rs8099917 and their corresponding nucleotide position are indicated (red). Adapted from [38, 39].

they could influence transcription factor binding and DNA methylation. However, while some studies detect a correlation of protective IFNL SNPs and higher IFNL expression levels, other fail to do so (see [39] for detailed description). Mechanistically, the rs8099917 SNP has been suggested to influence IFNL3 mRNA stability with the favorable allele being more stable [118]. For the rs368234815(TT/ΔG) SNP the functional impact is best described [4]. The ΔG variant results in the expression of IFNL4, which is a pseudogene in TT carriers. IFNL4 expression is associated with increased ISG levels and this in turn worsens treatment outcome. While this might seem counterintuitive, it is in line with observations that patients with increased pretreatment ISG levels in the liver respond more poorly to IFN-alpha/RBV therapy [119–121]. Thus, it seems at least in the case of IFNL4 that it has an adverse effect during hepatitis C by desensitizing the liver to IFN-alpha/RBV treatment. This has been confirmed in an independent study on the IFNL4 coding SNP rs117648444 [90], which results in a less active IFNL4 variant and consequently in improved treatment response. While one might question the value of these genetic markers in the age of IFN-free DAA treatment with high cure rates, it should be noted that IFNL locus SNPs are also predictive for DAA treatment outcome and moreover influence the DAA response kinetics [122–124]. Genetic markers might therefore allow prediction of treatment duration and consequently reduce costs and exposure time to DAAs.

3.2.2. Association with Spontaneous HCV Clearance. Human polymorphisms in the IFNL locus responsible for improved response to IFN-alpha/RBV treatment are also associated with better spontaneous clearance of HCV.

Allele frequency of the rs12979860 SNP differs between individuals with European or African ancestry with the favorable rs12979860(C) variant predominating in the former population. This finding correlates with better clearance of HCV in European ancestry individuals. The rs368234815(TT/ΔG) SNP similarly predicts HCV clearance rates. However, it is a better predictor than the rs12979860 SNP in African-Americans, while the predictive value of both SNPs is similar in European-Americans [4, 125]. Causative for this difference is the degree of linkage disequilibrium between both SNPs in the two populations [116]. The third SNP with strong predictive value (rs8099917) for the outcome of HCV infection also shows a higher frequency of the protective allele (T) in

individuals of European and Asian ancestry as compared to individuals of African ancestry.

In summary, there is a clear link between IFNL gene SNPs and HCV treatment outcome as well as spontaneous clearance. Notably, except for the SNP resulting in IFNL4 expression, the mechanisms causing the association remain elusive and the associated SNPs may not be the true causal variants. Nonetheless, the predictive value of the IFNL SNPs extends beyond hepatitis C as genetic associations with nonalcoholic fatty liver disease, allergy, and infection with cytomegalovirus, human T-lymphotropic virus, hepatitis B virus, HIV, and herpes simplex virus have been suggested (reviewed in [126]). The discovery of IFNL polymorphisms in the context of HCV infection may therefore importantly contribute to the understanding of other hepatic and extrahepatic diseases.

3.3. IFNL Therapy. Before the rise of DAAs targeting HCV, IFNLs were considered an attractive alternative to IFN-alpha/RBV treatment for several reasons. The antiviral profile of IFNL resembles the one of IFN-alpha, as both interferons signal via ISGF3. This holds true in primary human hepatocytes [105] as well as in hepatoma cell lines [49]. However the kinetics and magnitude of ISG induction differ between IFN-alpha and IFNL [55, 103, 127]. Another difference between the two IFN types is their tissue specificity caused by the divergent expression pattern of their receptors; in contrast to the IFNL receptor complex, which shows a restricted tissue expression, the IFN-alpha receptor is expressed ubiquitously. Thus compared to IFNL, IFN-alpha acts more systemic, causing more adverse effects, which are often limiting treatment options and compliance.

The overlapping response of IFN-alpha and IFNL signaling on the one hand and the tissue specificity of the IFNLR on the other hand made IFNL promise that the replacement of IFN-alpha by IFNL would yield the same therapy outcome with fewer side effects.

Indeed, clinical studies revealed an improved safety and tolerability profile for PEG-IFNL1a compared to PEG-IFN-alpha [128, 129]. When used in combination with RBV and the DAA Daclatasvir, a 24-week PEG-IFNL1a based treatment does not only show less adverse events, but also leads to a higher sustained virological response than treatment with a PEG-IFN-alpha based regime; 12 weeks after treatment no HCV RNA is detectable in the blood of 88% of patients in the PEG-IFNL1a group compared to only 70.5% of patients in the PEG-IFNL1a group [130]. In a different clinical study PEG-IFNL1a or PEG-IFN-alpha2a were given together with RBV and Telaprevir, but here no noninferiority of PEG-IFNL1a regarding safety, tolerability, and efficacy was observed [131].

IFNL3 has the highest activity among the IFNL types and therefore might be more suitable as therapeutic agent than IFNL1 [55]. Nevertheless, only IFNL1 has been evaluated in clinical trials so far, probably due to the fact that recombinant IFNL3 is difficult to produce. Recently IFNL3 analogs, which allow high yield production and are comparable to IFN-alpha2a in their ability to inhibit HCV replication in Huh-7.5.1 cells, have been designed [132]. However, the licensing of effective DAA paved the way for an IFN-free therapy of CHC, which is becoming the standard of care nowadays. Thus most likely IFNL will not be needed as therapeutic agent for hepatitis C in the future.

While the development of hepatitis C drugs is fortunately an unprecedented success story [133], we still lack specific drugs for other hepatotropic viruses. For instance, hepatitis E virus is sensitive to IFNL in in vitro models [134] and currently treatment options for hepatitis E are limited. Consequently, the mechanistic insights on the interplay of IFNL and HCV might spur important future work on the role and possible therapeutic application of IFNL during infection with other viruses infecting IFNLR expressing tissues, in particular the liver and the lung.

Competing Interests

The authors have no competing interests.

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Research Article

Novel Chemokine-Based Immunotoxins for Potent and Selective Targeting of Cytomegalovirus Infected Cells

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Immunotoxins as antiviral therapeutics are largely unexplored but have promising prospective due to their high selectivity potential and their unparalleled efficiency. One recent example targeted the virus-encoded G protein-coupled receptor US28 as a strategy for specific and efficient treatment of human cytomegalovirus (HCMV) infections. US28 is expressed on virus-infected cells and scavenge chemokines by rapid internalization. The chemokine-based fusion-toxin protein (FTP) consisted of a variant (F49A) of CX_3CL1 specifically targeting US28 linked to the catalytic domain of *Pseudomonas exotoxin* A (PE). Here, we systematically seek to improve F49A-FTP by modifications in its three structural domains; we generated variants with (1) altered chemokine sequence (K14A, F49L, and F49E), (2) shortened and elongated linker region, and (3) modified toxin domain. Only F49L-FTP displayed higher selectivity in its binding to US28 versus CX_3CR1, the endogenous receptor for CX_3CL1, but this was not matched by a more selective killing of US28-expressing cells. A longer linker and different toxin variants decreased US28 affinity and selective killing. Thereby, F49A-FTP represents the best candidate for HCMV treatment. Many viruses encode internalizing receptors suggesting that not only HCMV but also, for instance, Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus may be targeted by FTPs.

1. Introduction

Immunotoxins constitute a class of protein-based therapeutics and are considered promising anticancer therapies in the clinic [1, 2]. They are chimeric molecules that consist of a toxin fused to a targeting moiety. The targeting domain is most commonly the antigen-binding fragment of a monoclonal antibody but can also comprise receptor ligands, such as a growth factor or a cytokine that targets specific cell surface receptors [1]. *Pseudomonas* exotoxin A (PE) is a highly toxic protein that has been used to generate several immunotoxins undergoing evaluation in clinical trials [3–5]. The structure and mechanism of action of PE allow for modifications so that PE can be converted into an immunotoxin by changing its target to a different cell surface receptor [6]. PE-based immunotoxins usually contain a fragment of the *Pseudomonas aeruginosa* exotoxin A, consisting of domains II and III of the native toxin, while domain I is replaced by the targeting moiety. Once the target domain binds to its receptor, the immunotoxin is internalized by endocytosis, cleaved in domain II by the proprotein convertase furin, leading to the release of the toxin and a subsequent cell killing.

Within the last decades, the potential and success rate of developing anticancer immunotoxins have been translated to other indications, such as infectious diseases [1]. Recently, the first antiviral immunotoxin entered the stage, targeting the viral G protein-coupled receptor (GPCR) US28 encoded

by the human cytomegalovirus (HCMV) [7]. The targeting moiety was not an antibody, but a variant of the chemokine CX_3CL1 optimized for specific binding to US28. Wild- type CX_3CL1 only targets one additional receptor, namely, its cognate receptor, CX_3CR1. CX_3CL1 is unique among endogenous chemokine ligands, as it exists in two forms: a soluble form and a form where the chemotactic chemokine is anchored to the cell membrane by an extended mucin-like stalk and an alpha-helix through the membrane [8].

Viral piracy of the endogenous chemokine system is a commonly used viral strategy to circumvent and/or manipulate the host chemokine system and thereby the host immune response in benefit of virus survival and spreading [9–11]. Thus, HCMV devotes a significant part of its genome to immune modulatory gene homologs, including several predicted 7-transmembrane GPCRs: UL33, UL78, US27, and US28, with US28 being a functional chemokine G protein-coupled receptor [12]. Still related to the immune system but outside the chemokine system, herpesviruses have developed another strategy to manipulate the host by downregulation of surface expressed MHC class I molecules, a property described for the constitutively active GPCR denoted BILF1 by Epstein-Barr virus (EBV) [13–18]. However, most of the viral GPCRs show homology to the humane chemokine receptors, for example, the resemblance of US28 to the human CX_3CR1 receptor [9, 12] and the CXC-chemokine receptors encoded by rhadinoviruses like ORF74 encoded by human Kaposi's sarcoma-associated herpesvirus (KSHV) and of *Equine herpesvirus* 2 and ECRF3 encoded by *Herpesvirus saimiri* [19–22]. In addition, viruses encode chemokine ligands, for example, vCCL1-3 encoded by KSHV [23] and MC148 from the pox virus molluscum contagiosum [24–26]. A third principle to target the chemokine system by virus is by scavenging host chemokines by viral chemokine binding proteins [27].

US28 is a broad-spectrum chemokine receptor yet binds CX_3CL1 with superior affinity as compared to CC-chemokines [28]. Moreover, it signals with high constitutive activity [29, 30] and undergoes constitutive ligand-independent receptor internalization [31], a feature well suited for immunotoxin delivery. Based on the molecular and pharmacological properties of US28 and the structural property of CX_3CL1, the prototype immunotoxin CX_3CL1-FTP was designed [7]. The chemokine domain of CX_3CL1 was chosen as targeting moiety and the mucin-like stalk of CX_3CL1 was replaced by catalytic active domains of PE. Moreover, as CX_3CL1 also binds to the human CX_3CR1, a US28 selective FTP was generated (F49A-FTP) (Figure 1), by introducing a single point mutation (Phe^{49} to Ala) in CX_3CL1 [7]. Both FTPs were highly efficient in controlling HCMV infections in vitro and F49A-FTP provided unparalleled potency compared to the gold standard ganciclovir in vivo [7].

These promising results suggest that chemokine-based FTPs can be developed into therapeutics to treat HCMV-associated diseases. Here, we investigate if the US28 selective FTP (F49A-FTP) can be further optimized in terms of increased selectivity or potency by a systematic approach modifying the US28-targeting part (i.e., the chemokine), the catalytic active domains of PE, and alternations in the linker region.

2. Materials and Methods

2.1. Antiviral Fusion-Toxin Proteins (FTPs). The FTPs were prepared as described previously [7]. Briefly, the FTP DNA constructs were cloned into the pET21a(+) vector (Novagen) and transformed into *E. coli* BL21(DE3) pLysS cells (Novagen) for the preparation of inclusion bodies. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were ruptured by sonication in the following buffer: 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 10 mM $MgCl_2$, 1 mM benzamidine, 3 mM DTT, 1 mM PMSF, and 10 μg/mL DNase I. The suspension was centrifuged and the pellet was resuspended and washed twice with 50 mM Tris-Cl pH 8.0, 300 mM NaCl, 0.25% sodium deoxycholate, and 5 mM DTT (the second time without sodium deoxycholate).

2.1.1. Downstream Purification of Recombinant FTPs. 100 μL denaturation buffer (3 M GnHCl, 100 mM Tris-Cl pH 8.0, 5 mM EDTA, and 5 mM DTT) was added to solubilize the inclusion bodies, followed by incubation and centrifugation. The inclusion bodies were dialyzed against 1x PBS at 4°C and then overnight against 1x PBS containing 0.2 mM cystine and 1 mM cysteine. The protein sample was added to two sample volumes of 50 mM Tris-Cl pH 8.0 while stirring, followed by addition of 3 sample volumes of 50 mM HEPES pH 7.2, 1 mM $CaCl_2$, and 5 mM $MgCl_2$ and confirmation of the mixture reaching pH 7.2. The sample was filtered and loaded onto a Source 30Q column equilibrated in buffer A: 50 mM HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 50 mM NaCl. Bound protein was eluted with a gradient from 0 to 40% buffer B; buffer B is the same as buffer A, but with addition of NaCl to 1 M, and the fractions with the protein of interest were concentrated. The sample was centrifuged and loaded onto a Superdex 75PG column, equilibrated in 1x PBS.

2.2. Tissue and Virus Culture. Human lung fibroblasts cells MRC-5 (ATCC CCL-171) were purchased from the American Type Culture Collection (ATCC). The stable inducible clones of US28 and CX_3CR1 were kindly provided by Hjortø et al. (Department of Neuroscience and Pharmacology, University of Copenhagen) [32]. MRC-5 cells were grown at 10% CO-2 and 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 180 units/mL penicillin. The stable clones of inducible US28 and CX_3CR1 HEK-293 cells were grown as previously described [32]. The recombinant $Toledo_{LUC}$ strain was kindly provided by Dulal et al. (Department of Microbiology and Molecular Genetics, Rutgers-New Jersey Medical School) [33]. $Toledo_{LUC}$ virus stocks were propagated using MRC-5 cells and titrated as described previously [33].

2.3. Radioligand Competition Binding Assay. Stable inducible clones of US28 and CX_3CR1 cells were seeded at 10,000

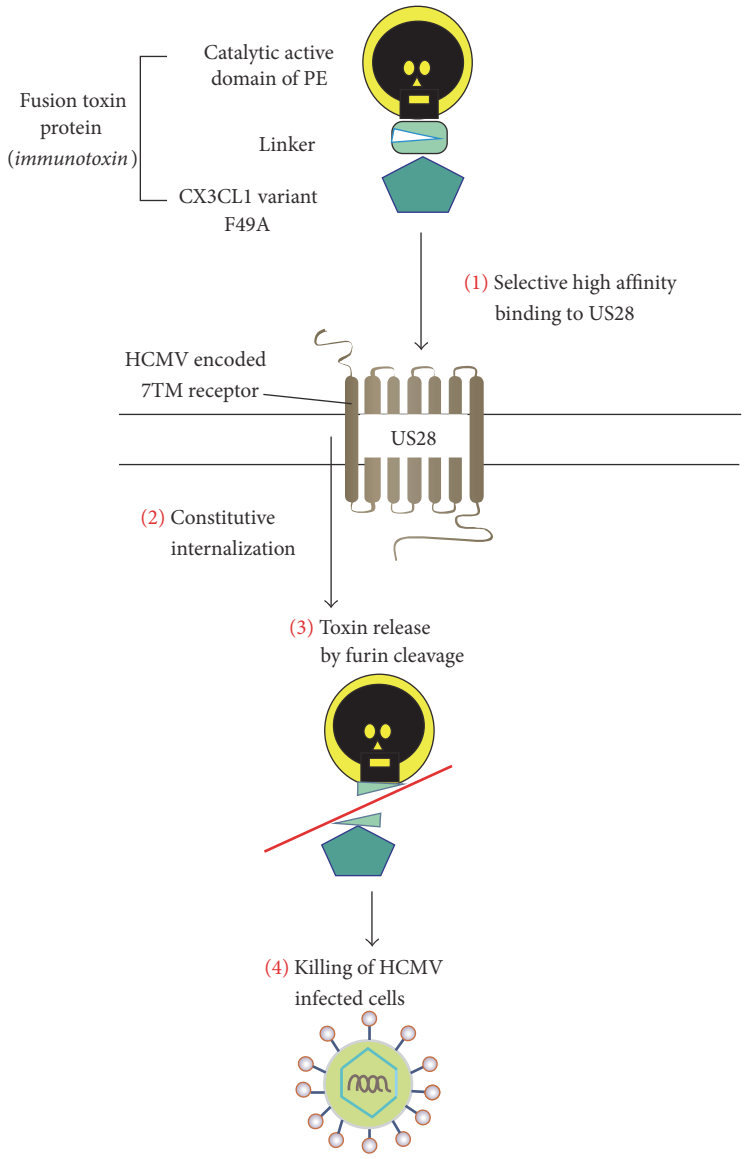

Figure 1: Selective killing of HCMV infected cells by F49A-FTP. The FTP consisting of the CX_3CL1 variant F49A and the catalytic active domains of PE binds selectively to US28 (1), and the internalization of the FTP is triggered by internalization of F49A binding to US28 (2). The release of F49A is achieved by furin cleavage (3), and the protein synthesis is inhibited by PE, leading to (4) killing of the human cytomegalovirus (HCMV) infected cells.

cells/well in poly-D-lysine (Invitrogen) coated 96-well tissue culture plates (Nunc). One day after seeding US28 and CX_3CR1 receptor expression was induced by tetracycline (Invitrogen; 3,6 ng/mL and 5 ng/mL, resp.) aimed at obtaining 5–10% specific binding of the added radioactive ligand. One day after induction, cells were assayed by competition binding for 3 h at 4°C using 20–70 pM ^{125}I-CX_3CL1 as well as unlabeled ligand 10 pM to 100 nM in 50 mM Hepes buffer pH 7.4, supplemented with 1 mM $CaCL_2$, 5 mM $MgCL_2$, and 0,5% (w/v) bovine serum albumin (BSA) (binding buffer). After incubation, cells were washed twice in ice-cold binding buffer and supplemented with 0,5 M NaCl. Determinations were made in quadruplicate.

2.4. Cell Killing Assay. Stable clones of inducible US28 and CX_3CR1 HEK-293 cells were seeded in poly-D-lysine-coated 48-well tissue culture plates (Nunc) in 300 μL DMEM (Invitrogen). One day after seeding US28 and CX_3CR1 receptor expression was induced by tetracycline (0,125 μg/mL and 0,25 μg/mL, resp.). One day after induction, a single dose treatment was applied with indicated concentrations of the FTPs (10 pM to 100 nM) and buffer (negative control) and cells were incubated for 24 h at 37°C. To estimate cell health, the cells were incubated with AlamarBlue (Invitrogen) in DMEM without FBS (10% solution) 300 μL per well, for 4 h at 37°C. Data were collected using a Synergy HT plate reader. Determinations were made in quadruplicate.

Table 1: Homologous binding experiments testing the binding affinity of the FTPs.

	US28/^{125}I-CX_3CL1^{**}		*CX_3CR1*/^{125}I-CX_3CL1^{**}		Binding selectivity
	log IC_{50} ± SD	IC_{50} [nM]	log IC_{50} ± SD	IC_{50} [nM]	CX_3CR1 versus US28 (CX_3CL1)
CX_3CL1-FTP	−8.9 ± 0.26	1.3	−7.3 ± 0.45	50	**40**
K14E-FTP	−7.2 ± 0.31	63	*−6.2 ± 0.63*	*631**	***10***
F49A-FTP	−7.5 ± 0.59	32	*−5.6 ± 0.81*	*2512**	***79***
F49L-FTP	−8.2 ± 0.95	6.3	*−5.5 ± 0.36*	*3162**	***501***
F49E-FTP	−6.5 ± 0.07	316	*−5.3 ± 0.40*	*5012**	***16***
F49A-FTP 2	−7.4 ± 0.49	40	*−6.9 ± 0.50*	*126**	***3***
F49A-FTP 3	−7.3 ± 0.64	50	*>5,0*		—
F49A-FTP 4	−7.6 ± 0.24	25	*−6.5 ± 0.21*	*316**	***13***
F49A-FTP 7	−7.3 ± 0.38	50	*−6.6 ± 0.20*	*251**	***5***

* IC_{50} values of the FTPs bound to CX_3CR1 have been estimated from a partial curve.
** IC50 value estimated from 3-4 experiments.

2.5. HCMV Luciferase Assay. MRC-5 (ATCC CCL-171) were seeded in 96-well white tissue culture plates (Nunc) at an initial cell density of 8000 cells/well and infected the next day with $Toledo_{LUC}$ (multiplicity of infection of 0,1 [1 out of 10 cells]). The day after, cells were treated with a single dose of different concentrations of FTPs (10 pM to 1 μM) and buffer (negative control), followed by an incubation for three days at 37°C. On day four after infection, media were exchanged and 100 μL 1x PBS supplemented with $MgCl_2$ and $CaCl_2$ as well as 100 μL britelite™ plus reagent (Perkin & Elmer) were added. Luciferase activity was measured using a Synergy HT plate reader. Determinations were made in quadruplicate.

2.6. Data Analyses. Data analyses were performed using Prism version 6.0.1. Data are expressed as means ± SEM.

3. Results

3.1. Modification in the Chemokine Domain to Gain More Selectivity for US28. Based on affinity determination of 35 variants of CX_3CL1 on US28 and CX_3CR1, we previously identified F49A as the CX_3CL1 variant with the highest selectivity to US28 versus CX_3CR1 (affinity selectivity index of 182 determined as IC_{50} for CX_3CR1 relative to IC_{50} for US28) [7]. Two other CX_3CL1 variants (K14E and F49L) also turned out to be selective towards US28 with a selectivity index of 39 and 81, respectively, and both maintained high affinity for US28 [7]. Based on these results, the two recombinant CX_3CL1 variants were fused to PE to create the new fusion-toxin proteins: K14E-FTP and F49L-FTP (Figure 2(a)). As the Ala-substitution of Phe49 resulted in the highest selectivity index, we further explored this position, by the introduction of a glutamic acid, and fused this chemokine with the toxin to create F49E-FTP. The three FTPs were tested for binding to US28 and CX_3CR1 using ^{125}I CX_3CL1 as radioligand and compared to CX_3CL1-FTP (the "prototype") and F49A-FTP (Figure 2(b)). F49L-FTP maintained high affinity for US28, as the affinity to US28 was 5 times increased compared to F49A-FTP (Figure 2(c) and Table 1) [7]. In contrast, F49L-FTPs affinity to CX_3CR1 was low (in the millimolar range), leading to a selectivity index of 501 (Figure 2(c) and Table 1). Thus, F49L-FTP displayed a ~6,3-fold higher selectivity for binding to US28 relative to CX_3CR1 compared to F49A-FTP. However, when testing the cell killing activity of the FTPs (Figure 2(d)), F49A-FTP was still more selective in killing US28- versus CX_3CR1-expressing cells with a 513-fold higher potency on US28- versus CX_3CR1-expressing cells, whereas F49L-FTP was half as selective with a 275-fold higher potency (Figure 2(e), Table 2). Despite the overall higher binding affinity to US28 as compared to CX_3CR1 of F49L-FTP, the FTP with the best selectivity profile in killing US28-expressing cells (F49A-FTP) was chosen as lead candidate for further optimization of the nonchemokine parts.

3.2. Refinement of the Linker Region with Parts of the Mucin-Like Stalk of CX_3CL1. Three FTPs with an extended linker were obtained by adding variable lengths of the mucin-like stalk of CX_3CL1 (F49A-FTP-2 [9aa], F49A-FTP-3 [21aa], and F49A-FTP-4 [41aa]) (Figure 3(a)). The FTPs maintained high binding affinity to US28 similar to F49A-FTP (Figure 3(b)) but had a reduced selectivity in their binding affinity to US28 versus CX_3CR1 (Figure 3(c)), as their affinities to CX_3CR1 increased (Figure 3(b)). Furthermore, the three FTPs had reduced potencies in cell killing of US28-expressing cells proportional to the length of the added mucin-like stalk domain (Figure 3(d)). F49A-FTP-3 and -4 with the longest part of the mucin-like stalk had an up to 3.3-fold lower selectivity in killing US28- versus CX_3CR1-expressing cells compared to F49A-FTP (Figures 3(d) and 3(e); Table 2). Taken together, these results show that elongation of the linker region with parts of the mucin-like stalk decreases the potency and selectivity of the FTPs compared to F49A-FTP.

3.3. Reducing the Linker Region by Removing Domain II of Pseudomonas Exotoxin (PE). The furin cleavage site, located in domain II of PE, is important for cleavage of the cytotoxic domains of PE from the chemokine part. We designed two FTPs F49A-FTP-5 and F49A-FTP-6 without domain II and hence without the furin cleavage site. In F49A-FTP-6, we inserted an additional three-amino-acid linker (Gly, Gly, and Ser (GGS)) between the chemokine domain of CX_3CL1 and the Ib/III domains of PE (Figure 4(a)). The F49A-FTP-5

Table 2: Cell killing activity of the FTPs.

	*US28 exp. cells***		*CX_3CR1 exp. cells***		Selectivity
	log IC_{50} ± SD	IC_{50} [nM]	log IC_{50} ± SD	IC_{50} [nM]	US28 versus CX_3CR1
CX_3CL1-FTP	−10.6 ± 0.52	0.026	−9.6 ± 0.45	0.25	**10**
K14E-FTP	−10.5 ± 0.25	0.029	−8.0 ± 0.22	11	**380**
F49A-FTP	−10.6 ± 0.24	0.028	−7.8 ± 0.15	14	**513**
F49L-FTP	−11.1 ± 0.41	0.008	−8.7 ± 0.24	2.2	**275**
F49E-FTP	−9.6 ± 0.47	0.27	*−7.5 ± 0.30*	*32**	***117***
F49A-FTP 2	−10.3 ± 0.08	0.051	*−7.8 ± 0.30*	*16**	***309***
F49A-FTP 3	−9.8 ± 0.16	0.15	*−7.6 ± 0.20*	*25**	***162***
F49A-FTP 4	−9.8 ± 0.06	0.17	*−7.6 ± 0.30*	*25**	***151***
F49A-FTP 5	n.a.		n.a.		—
F49A-FTP 6	−9.5 ± 0.20	0.34	*−7.5 ± 0.10*	*32**	***93***
F49A-FTP 7	−11.3 ± 0.24	0.005	−9.5 ± 0.49	0.35	**66**

*log IC50 values are estimated from a partial curve.
**IC50 value estimated from 3–6 experiments.

variant had a reduced antiviral activity, whereas that of F49A-FTP-6 was unchanged compared to F49A-FTP (Figures 4(b) and 4(c); Table 3). As selective killing of US28-expressing cells by the FTP is essential in order to minimize unwanted killing of uninfected host cells expressing CX_3CR1, we tested F49A-FTP-6-mediated killing of US28- versus CX_3CR1-expressing cells. Compared to F49A-FTP, F49A-FTP-6 displayed a reduced selectivity for killing US28-expressing cells, as it was ~5.5 times less selective (Figures 4(d) and 4(e) and Table 2). In summary, the domain II of PE is not essential for the antiviral activity, yet its removal decreases the selective killing of the FTPs indicating an altered function of the FTP.

3.4. Elongating the Linker with a Full Catalytic Domains of PE. As a final step of this study, we investigated if we could increase the antiviral activity of the FTP by fusion of the chemokine with the full catalytic active domains of PE (domains II, Ib, and III) in variant 7, F49A-FTP-7 (Figure 5(a)). This variant lost selectivity by having a much higher potency (40-fold) in killing CX_3CR1-expressing cells compared to F49A-FTP (Figure 5(b)) and only a slightly higher potency (5,6 fold) in killing US28-expressing cells. The FTP was thereby 7,7 times less selective in killing US28- versus CX_3CR1-expressing cells with a selectivity index of 66 compared to 513 for F49A-FTP (Table 2). We further determined the antiviral activity of F49A-FTP-7 (Figure 5(c)) and consistent with its improved cell killing, it displayed a higher potency (up to 7,6 times) compared to F49A-FTP. In summary, changes in the enzymatic domains of PE led to a higher antiviral activity of the FTPs but also to more unspecific killing of CX_3CR1-expressing cells. Based on the results, the prototype FPT "F49A-FTP" with the selective chemokine binding domain and the truncated enzymatic domains (translocation domains II and Ib) turned out to be the best FTP to control virus infections and superior selectivity in killing US28-expressing cells compared to all tested FTPs.

Table 3: Antiviral activity of the FTPs.

	Potency*	
	log IC_{50} ± SEM	IC_{50} [nM]
CX_3CL1-FTP	−9.3 ± 0.36	0.52
K14E-FTP	−8.1 ± 0.09	8.9
F49A-FTP	−7.7 ± 0.05	20
F49A-FTP 2	n.a.	n.a.
F49A-FTP 3	n.a.	n.a.
F49A-FTP 4	n.a.	n.a.
F49A-FTP 5	−7.5 ± 0.24	30
F49A-FTP 6	−8.0 ± 0.10	9.3
F49A-FTP 7**	−8.6 ± 0.13	2.6

*IC50 value estimated from 3–5 experiments.
**IC50 value estimated from 2 experiments.

4. Discussion

In this study, we generated novel FTPs with the attempt to improve the previously published F49A-FTP [7] in terms of selective killing of US28-expressing cells and antiviral activity. In a systematic approach, we first optimize the chemokine part (that binds to the target receptor). We next varied the linker part (between the chemokine and the toxin) and finally the toxin (variations of PE). F49A-FTP was originally designed based on the selectivity profile (affinity of US28 versus CX_3CR1) of the chemokine part alone, which, after selection, was fused to the catalytic active domains of PE (Figure 1) [7]. The chemokine system is characterized by redundancy and promiscuity with chemokines that bind more than one receptor and vice versa, but there are also highly selective and monogamous receptor : ligand pairs such as CX_3CR1 : CX_3CL1 [34]. For CX_3CL1 that only binds to one endogenous receptor CX_3CR1 in addition to the viral US28, it is less complex to employ a rational design strategy to remove binding to the endogenous receptor CX_3CR1 compared to

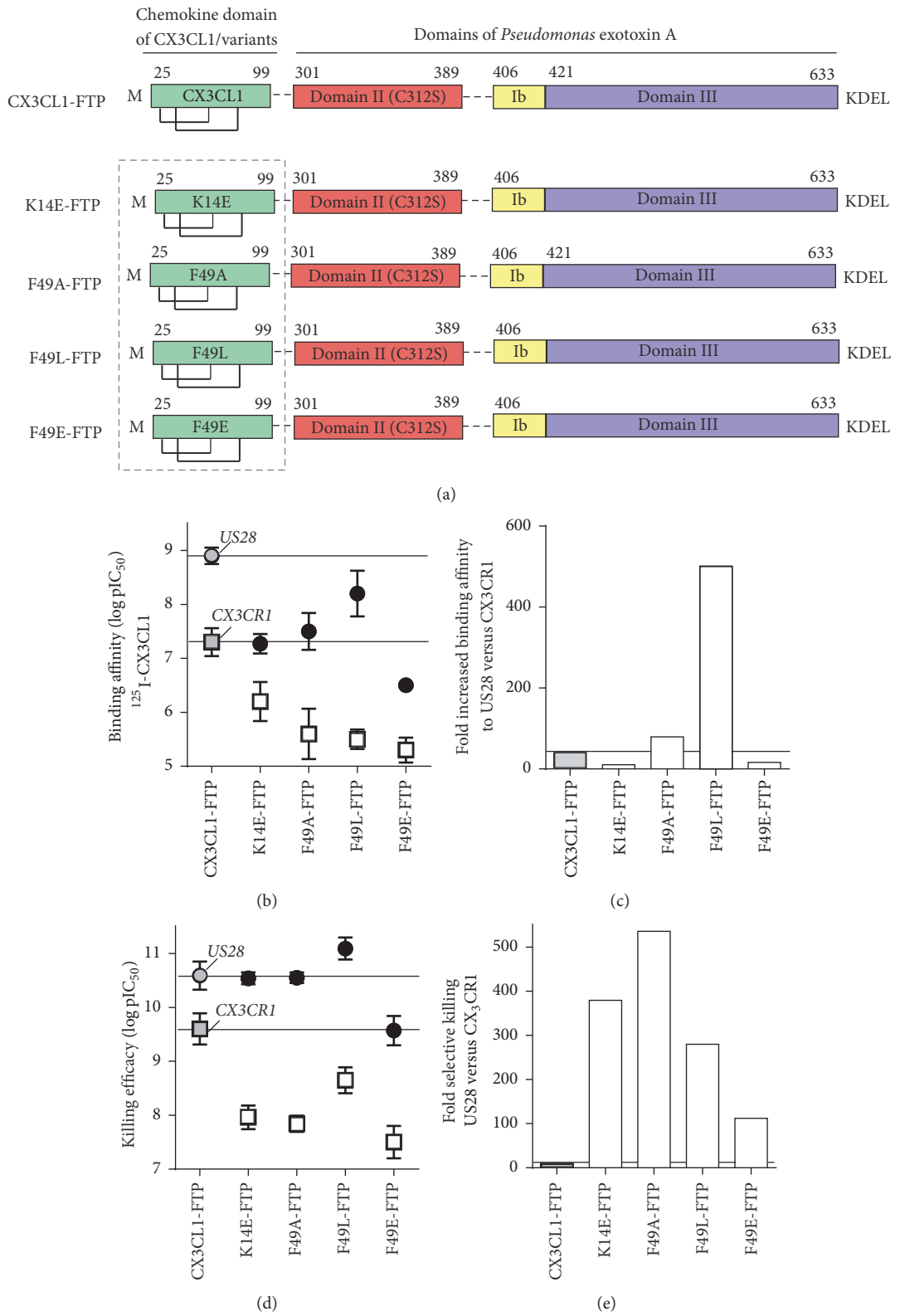

Figure 2: Design, binding, cell killing, and antiviral activity of FTPs with a modified CX_3CL1 domain. (a) Schematic representation of CX_3CL1-based FPTs created by fusion of CX_3CL1 variants to domains of PE. (b) Binding of the prototype CX_3CL1-FTP (grey symbols) and CX_3CL1-based FTPs on HEK-293 cells induced to express US28 (black circles) and CX_3CR1 (white squares). (c) Binding selectivity of CX_3CL1-FTP and CX_3CL1-based FTPs determined as fold improved affinity for US28 relative to CX_3CR1. (d) Cell killing of CX_3CL1-FTP (grey symbols) and CX_3CL1-based FTPs on tetracycline induced HEK-293 cells expressing US28 (black circles) and CX_3CR1 (white squares). (e) Selectivity of CX_3CL1-FTP and CX_3CL1-based FTPs determined as fold improved potency in killing US28- relative to CX_3CR1-expressing cells. Values present IC_{50} values from 3–5 independent biological replicates (b) and (d).

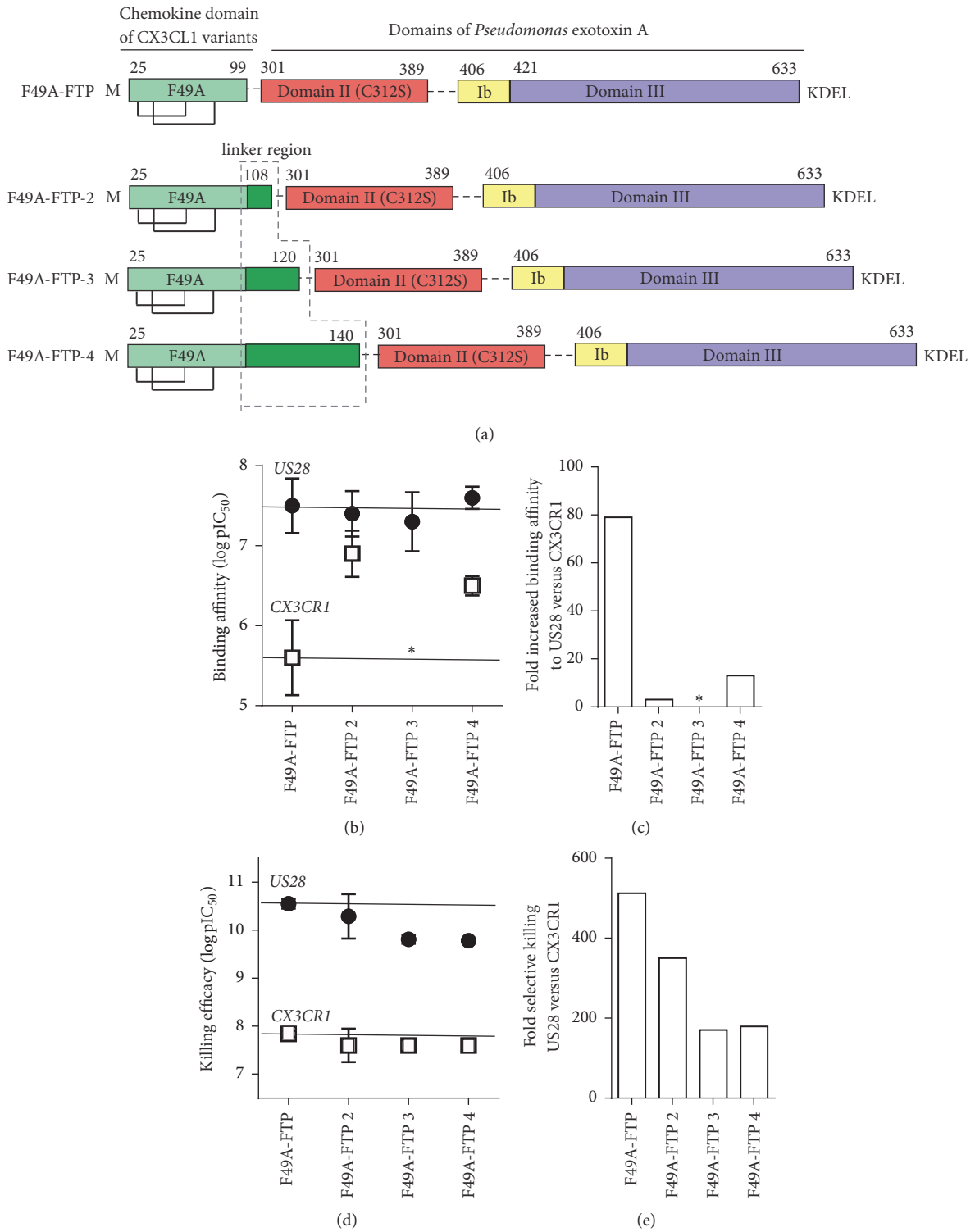

Figure 3: Design, binding, and cell killing of FTPs containing parts of the mucin-like stalk of CX_3CL1. (a) FTPs with an extended linker consisting of parts of the mucin-like stalk of CX_3CL1. (b) Binding of F49A-FTP and FTPs from this group on HEK-293 cells induced to express US28 (black circles) and CX_3CR1 (white squares). The IC_{50} value for F49A-FTP-3 is $>10^{-6}$ M (no binding detectable on CX_3CR1 expressing cells; marked with a star) and the binding selectivity was therefore not analyzed in (c). (c) Binding selectivity determined as fold improved affinity for US28 relative to CX_3CR1. (d) Cell killing of F49A-FTP and FTPs from this group on tetracycline induced HEK-293 cells expressing US28 (black circles) and CX_3CR1 (white squares). (e) Selectivity of F49A-FTP and FTPs from this group determined as fold improved potency in killing US28- relative to CX_3CR1-expressing cells. Values present IC_{50} values from 3–5 independent biological replicates (b) and (d).

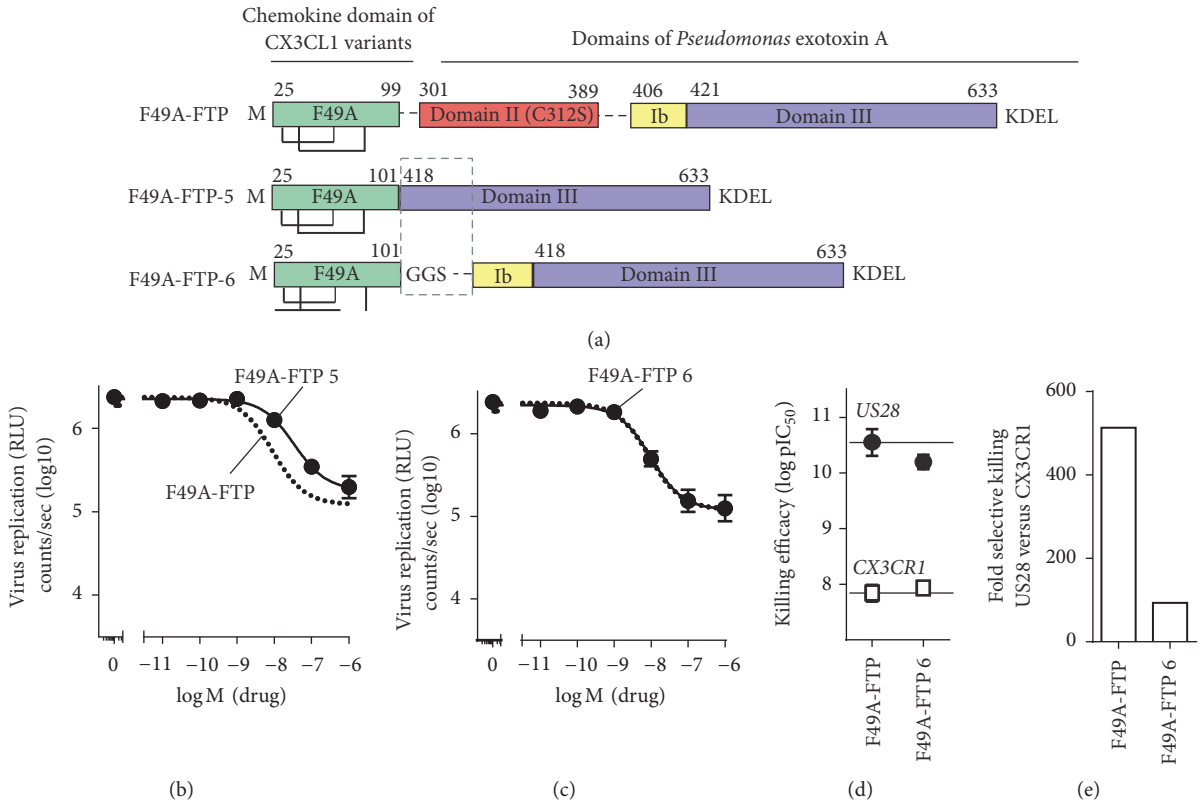

Figure 4: Design, cell killing, and antiviral activity of FTPs without domain II and optional with as GS-linker and Ib domain. (a) F49A-FTP as template for F49A-FTP-5 without domain II between the chemokine domain of CX_3CL1 and domain III of PE or for F49A-FTP-6 with an additional amino-acid linker (GGS) and part of the Ib domain. (b-c) Inhibition of virus replication measured by luciferase activity of MRC-5 cells infected with $Toledo_{LUC}$ (MOI 0,1) and treated once with F49A-FTP (pos. control; dotted line), F49A-FTP-5, and F49A-FTP-6 (black circles). (d-e) Selectivity of F49A-FTP and F49A-FTP-6 determined as fold improved potency in killing US28- relative to CX_3CR1-expressing cells. Error bars indicate SEM for 3–5 independent biological replicates.

the US28 binding CC-chemokines that interact with multiple endogenous CC-chemokine receptors [35, 36]. Importantly, the unique mucin-like stalk of CX3CL1 is not necessary for receptor binding as CX3CL1 with this elongation binds similar to CX3CR1 as the soluble chemokine domain of CX3C L1 does [37]. For US28, the affinity of CX3CL1 with its mucin-like stalk is even seven times lower than that of the CX3C chemokine domain alone [28]. However, as US28 still binds the full length CX3CL1 with high affinity (nM) [28], this chemokine is suitable for FTP development as the protein allows for C-terminal modifications and elongations with maintained US28 recognition (Figure 3(b)). Chemokine receptor binding is in general facilitated by interactions between the positively charged chemokine core and the negatively charged extracellular receptor domains, usually including the N-terminus [38, 39]. F49A was picked among 35 CX_3CL1 variants as the most selective candidate [7]. In the present study, we chose two other selective CX_3CL1 variants (F49L and K14E), in addition to an extra variant at position 49 (F49E) to create FTPs by fusion of the chemokine fragment with the *Pseudomonas* exotoxin fragments and compared their binding and killing properties to those of F49A-FTP. F49L-FTP had the highest selectivity index in terms of binding, yet due to an improved killing of CX_3CR1 expressing cells and maintained high killing of US28-expressing cells, the selectivity in terms of killing was reduced. Thus, to create a highly US28-selective FTP as F49A-FTP, it is essential not only to determine its binding affinity to the target receptor but also to investigate its specific killing property. A change of the naturally occurring ligand-receptor complex can influence the molecular properties of the receptor, for example, the rate of receptor internalization or its intracellular trafficking, that is, important receptor features for toxin delivery.

To control HCMV infections, selective targeting of the infected cells is required, but also a high efficacy of the FTP to kill cells before the virus spreads is required. Therefore, we further investigated if we could increase the efficacy of F49A-FTP by modifying the linker region or the catalytic active domains of PE. Elongation of the linker region with parts of the mucin-like stalk of CX_3CL1 and also deletion of domain II of PE reduced the efficacy of the FTPs in killing US28-expressing cells compared to F49A-FTP. This is consistent with previous studies where eliminating the furin cleavage site by deletion or preventing cleavage with a point mutation in the sites reduced the cytotoxicity of a series of immunotoxins [40].

Besides changes in domain II, changes in the full catalytic domains of PE (domains II, Ib, and III) increased the

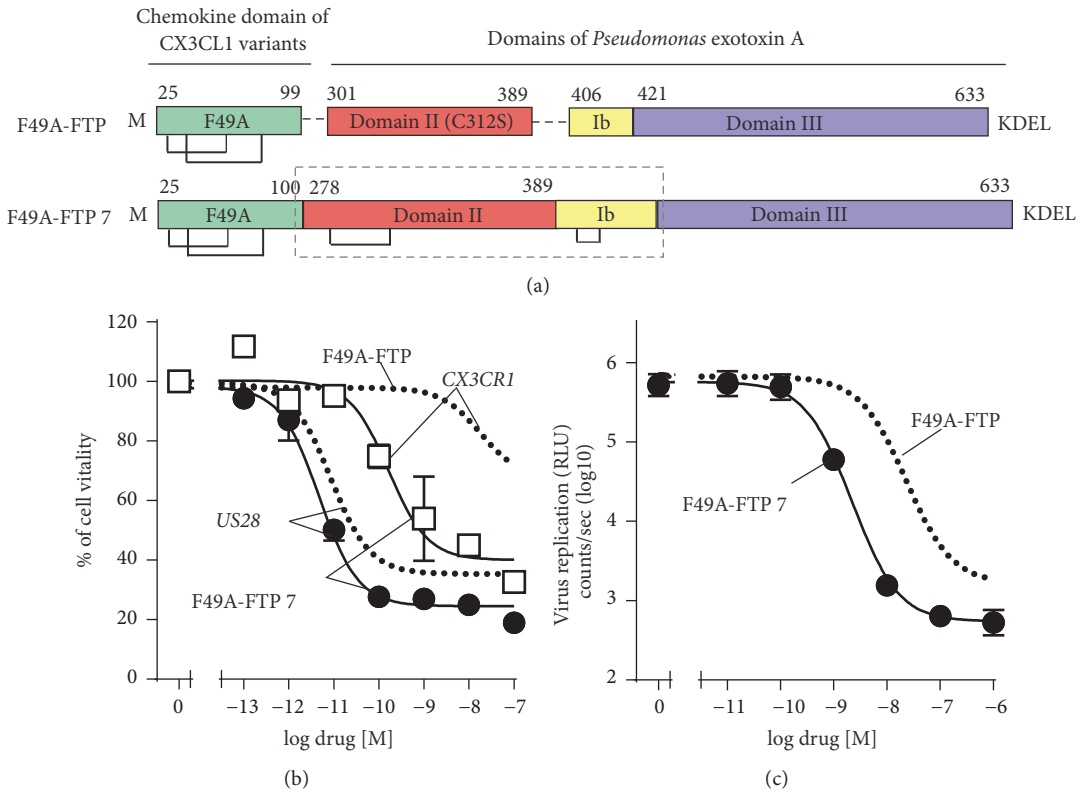

Figure 5: Design, cell killing, and antiviral activity of an FTP with full domains of PE. (a) F49A-FTP-7 with full catalytic active domains of PE. (b) F49A-FTP-7 induced cell killing in comparison to F49A-FTP (dotted line) on tetracycline induced HEK-293 cells expressing US28 (black circles) and CX_3CR1 (white squares). (c) Inhibition of virus replication measured by luciferase activity of MRC-5 cells infected with $Toledo_{LUC}$ (MOI 0,1) and treated once with F49A-FTP (dotted line) or FTPs from this group (black circles). Error bars indicate SEM for 2–5 independent biological replicates.

cytotoxicity and antiviral property of the FTP (F49A-FTP-7). However, the increased killing was observed in both US28- and CX3CR1-expressing cells, indicating a generally improved toxicity of the FTP, again consistent with previous studies [40]. As selective action towards US28 is a perquisite to avoid side effects from inadvertent killing of CX_3CR1-expressing cells, the prototype F49A-FTP still presents the best candidate therapeutic to treat HCMV infections. To sum up, the presented rational strategy of modifying the chemokine domain, the linker region, and the cytotoxic domains did not improve the prototype FTP. However, as the general understanding of PE toxicity is incomplete, more knowledge is needed. Moreover, questions remain regarding the binding and action of F49A-FTP. So far, the binding of the CX_3CL1 variants was tested in competition with CX_3CL1, but it remains to be determined if the modified CX_3CL1 domain of F49A-FTP can compete against the broad spectrum of CC-chemokines binding to US28, for instance, in an inflammatory situation. Moreover, it remains to be described how the CX_3CL1-based FTPs act on cells expressing the membrane bound CX_3CL1, as the soluble form of CX_3CL1 has been shown to bind to transmembrane CX_3CL1 with high affinity, that is, with the latter acting as a receptor that thereby influences the communication between cells [41].

Immunotoxins targeting virus-encoded receptors represent promising drugs, not only for HCMV therapy but also for other herpesviruses by targeting their virus-encoded GPCRs. EBV and KSHV infections can cause cancer and targeting of their GPCRs could be a novel anticancer treatment strategy. The broad-spectrum chemokine receptor ORF74 from KSHV thus seems highly suitable for immunotoxin targeting, as this receptor (1) can induce the onset of Kaposi's-like lesions [42, 43], (2) has a defined chemokine ligand profile [44, 45], and (3) is internalized in response to human CXCL-1 and -8 [46]. As such, using immunotoxins designed to target KSHV-infected cells could be a valid approach to efficiently kill KSHV-infected cells. With respect to the EBV-encoded BILF1 receptor, its pronounced cell surface expression [13, 47], constitutive internalization [17], and expression during the lytic virus replication cycle, but also in latency [48], indicate that this receptor is a promising drug target suitable for immunotoxin targeting delivery. A drawback is that EBV-BILF1 is an orphan receptor (i.e., with no known ligands), but new technologies including nanobody and monobody design could offer highly specific ligands for immunotoxin targeting of EBV-BILF1. In addition to EBV-BILF1, immunotoxin targeting of the endogenous receptor EBI2 [49, 50] that is upregulated upon infection with EBV could be a future strategy for anti-EBV treatment. The role of EBI2 in the EBV life cycle is still uncertain [51], but if EBV benefits from high EBI2 expression, then EBI2 could represent another drug target to control EBV-associated diseases. However, as EBI2

is not a viral protein, more side effects would be a risk factor as the receptor is expressed on a variety of immune cells (B-cells, T-cells, macrophages, dendritic cells, and many others) [52–54].

5. Conclusion

Immunotoxin based antiviral drugs offer a novel antiviral mechanism for combination therapy and for treating infections that have become resistant to the current first-line intervention. Here, we show that a CX_3CL1-based FTP targeting the HCMV-encoded GPCR US28 can be modified for highly effective killing of virus-infected cells by modifying the three structural domains of the FTP (the chemokine domain of CX_3CL1, the linker region, and the catalytic active domains of PE). By inserting single point mutations in the core domain of CX_3CL1, the FTP loses affinity for CX_3CR1, but not for the virus-encoded receptor US28. Changes in the linker region do not improve the activity of FTP, whereas changes in the catalytic active domains of PE increase the killing efficacy for US28-expressing cells and thereby the antiviral activity. Thus, CX_3CL1-based FTP can be used as scaffold to create highly efficient and selective FTPs to control HCMV infections. As several other virus-exploited GPCRs have been described, the principle of antiviral therapy targeting these receptors may not be limited to US28 for the targeting of HCMV but may be expanded to the targeting of ORF74 for KSHV treatment and BILF1 and/or EBI2 for the treatment of EBV-mediated diseases.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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